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MESTRADO EM MICROBIOLOGIA APLICADA

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Dissertação orientada pelos Profs. Doutores:

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Resumo

As leveduras das espécies *Saccharomyces bayanus* e *S. cerevisiae* são usadas em vários processos industriais, devido à sua capacidade fermentativa. Por isso, é importante que as leveduras sejam resistentes a elevadas concentrações de etanol, que é produzido pelas próprias durante a fermentação, de forma a que estes processos se tornem mais rentáveis.

As leveduras da espécie *S. cerevisiae* possuem vários sistemas que estão envolvidos na sua adaptação e tolerância ao etanol, sendo que alguns estão associados a mecanismos gerais de resposta a stress, enquanto outros são específicos para o stress de etanol[1]. Algumas destas respostas estão já bem caracterizadas, mas um estudo recente deixou algumas questões em aberto[2]. Mostrou que o etanol estimula a via da calcineurina/Crz1p, resultando numa tolerância a maiores concentrações de etanol. Mas, ficou por provar se, após o choque de etanol, existe um aumento da concentração de Ca^{2+} citosólico, e qual a sua origem[2]. Sendo assim, neste trabalho pretendeu-se contribuir com um conhecimento mais aprofundado acerca de como as leveduras respondem a um choque de etanol.

O primeiro passo consistiu na optimização dum protocolo que permitisse a detecção de variações na concentração de Ca^{2+} citosólico. O indicador fluorescente Fluo-4 AM foi introduzido no interior das células de levedura, utilizando-se a técnica de electroporação. Uma vez no interior da célula, os grupos éster são clivados por esterases intracelulares e a forma sensível ao Ca^{2+} é libertada. Quando o Fluo-4 AM se liga ao Ca^{2+} livre no citosol, ocorre um aumento da fluorescência[3,4], que é depois detectada por espectrofluorimetria. As condições de electroporação, nomeadamente a sua duração total (em número de milisegundos), voltagem e concentração de Fluo-4 AM utilizadas, foram optimizadas para ambas as espécies. No caso de *S. bayanus*, considerou-se que a melhor condição é a de 25 mseg de electroporação com 2500 V/cm, usando-se o Fluo-4 AM com uma diluição 1:2. No caso da estirpe tipo de *S. cerevisiae* a melhor condição é a de 10 mseg de electroporação com 2500 V/cm, sendo que o Fluo-4 AM deverá estar numa diluição de 1:8. Para as estirpes selvagem e mutantes de *S. cerevisiae* BY, a condição ideal de electroporação é de 25 mseg com 2500 V/cm, usando-se o Fluo-4 AM numa diluição 1:2. As condições consideradas óptimas foram posteriormente utilizadas nas restantes experiências.

Uma das principais conclusões deste estudo foi que as leveduras *S. bayanus* e estirpe tipo de *S. cerevisiae* respondem ao choque de etanol com um aumento da concentração de Ca^{2+} citosólico. Esta resposta é ainda mais intensa em *S. cerevisiae*, provavelmente devido à sua menor resistência natural ao etanol. Cruzando estes resultados com informação proveniente de estudos anteriores, o aumento dos níveis de Ca^{2+} citosólico vai resultar na formação de complexos Ca^{2+}/calmodulina, que irão activar a calcineurina. Por sua vez,

quando activada, a calcineurina desfosforila o factor de transcrição Crz1p, resultando na sua rápida translocação para o núcleo, onde é responsável pela expressão de genes que induzem a tolerância ao etanol[2].

Com base nos resultados obtidos para *S. bayanus* e estirpe tipo de *S. cerevisiae*, o Ca^{2+} envolvido nesta resposta parece ser proveniente principalmente dos reservatórios intracelulares (vacúolo). Mas, as experiências com as estirpes selvagem e mutantes de *S. cerevisiae* BY, apesar de confirmarem que as leveduras respondem ao choque de etanol com um aumento da concentração de Ca^{2+} citosólico, mostram ainda que o Ca^{2+} parece não só ser proveniente do vacúolo, mas também do meio extracelular. Coloca-se assim a hipótese de que o Ca^{2+} possa ter diferentes origens ao longo do processo de resposta ao choque de etanol, tal como já foi descrito para o stress hipotónico[5].

Os valores de intensidade de fluorescência correspondem a uma determinada concentração de Ca^{2+} citosólico, que foi determinada usando um kit de calibração com soluções padrão de Ca^{2+} livre em concentrações definidas. Assim, foi possível comprovar que os valores obtidos estavam, no geral, dentro da gama de concentração de Ca^{2+} citosólico esperada para estas espécies de levedura. Outra experiência permitiu ter a certeza de que o etanol não estava a interagir inespecificamente com o Fluo-4 AM, o que poderia levar a artefactos nos resultados de fluorescência. O etanol só por si, não tem efeito na fluorescência emitida pelo Fluo-4 AM, sendo que para ser registado um aumento na fluorescência, tem de existir um aumento da concentração de Ca^{2+}.

Vários estudos já mostraram que leveduras pré-expostas a uma quantidade não letal de etanol podem activar mecanismos de resposta ao stress que resulta numa resistência transiente a maiores concentrações de etanol[1]. Por essa razão, outro objectivo deste estudo consistia em investigar se o crescimento de ambas as espécies na presença de diferentes concentrações de etanol teria alguma influência no posterior aumento da concentração de Ca^{2+} citosólico, em resposta a um choque de etanol. No caso de *S. bayanus*, o crescimento na presença de 0, 3 ou 9% etanol (v/v) resultou em padrões de resposta semelhantes. Os resultados da estirpe tipo de *S. cerevisiae* mostram que, após crescimento com 3% de etanol no meio de cultura, parecem responder duma forma mais intensa ao choque de etanol, do que células que cresceram sem etanol.

Em *S. bayanus* e na estirpe tipo de *S. cerevisiae*, o etanol parecia actuar como um agonista do GPCR (*G-protein coupled receptor*) de detecção da glucose. Assim, os aumentos da concentração de Ca^{2+} citosólico detectados anteriormente neste estudo, poderiam dever-se à activação deste GPCR pelo etanol. Os resultados também sugerem que o etanol provavelmente pode actuar por uma via alternativa, além do GPCR, pela qual também promove o aumento da concentração de Ca^{2+} citosólico nestas espécies de levedura. Mas, os resultados obtidos com as estirpes selvagem e mutantes de *S. cerevisiae*

BY mostraram que afinal o etanol não está a actuar pelo GPCR de detecção da glucose, nem pelo GPCR de detecção de feromonas, porque a delecção dos genes de cada GPCR não eliminou o aumento da concentração de Ca^{2+} citosólico em resposta ao choque de etanol. Sendo assim, o etanol estará a actuar por uma via alternativa, que irá promover o aumento da concentração de Ca^{2+} citosólico nas leveduras.

Cerca de 30-40% de todas as drogas prescritas funcionam como agonistas ou antagonistas de GPCRs, e a maior parte dos GPCRs humanos são órfãos, ou seja, os seus ligandos ainda não são conhecidos. Portanto, esta é uma área actualmente muito promissora, pois estes receptores órfãos podem ser alvos para o desenvolvimento de novas drogas[6]. Apesar do etanol não parecer funcionar como agonista de nenhum GPCR destas espécies de levedura, o protocolo optimizado poderá ter aplicação em sistemas de detecção de fluorescência baseados em microchips, com o objectivo de acelerar e facilitar a detecção de novos agonistas e antagonistas de GPCRs. Inicialmente, tendo por base este protocolo, as leveduras podem ser utilizadas como controlo, para optimizar todo o sistema. Mas, no futuro, poderiam-se expressar GPCRs de mamíferos em leveduras, sendo para isso necessário substituir os GPCRs da via das feromonas, pelos GPCRs pretendidos[7]. Após modificações nas proteínas G e também no sistema de *output*, será possível testar rapidamente bibliotecas de ligandos, de forma a detectar quais activam determinado GPCR órfão. A utilização de leveduras em vez das células animais tem algumas vantagens, pois as primeiras são fáceis de crescer e manipular geneticamente, os custos envolvidos são baixos e são bastante mais resistentes[8].

Palavras-chave: Etanol; Cálcio; GPCR; Resposta a stress; *Saccharomyces* sp.

Abstract

Saccharomyces bayanus and *S. cerevisiae* are used in several industrial processes, mainly for their fermentation ability. It's important that yeasts can resist to high ethanol concentrations, produced during fermentation, in order to make these processes more profitable.

This work tried to contribute with a more detailed knowledge about how yeasts respond to an ethanol shock. One of the main conclusions was that both species respond to ethanol shock with an increase of cytosolic Ca^{2+} concentration, and this response is stronger in *S. cerevisiae* neotype strain. During this response, Ca^{2+} seems to come from extracellular media and intracellular stores. Crossing with previous studies results, the rise of cytosolic Ca^{2+} levels will result in the formation of Ca^{2+}/calmodulin complexes that will activate calcineurin. When activated, calcineurin dephosphorylates the transcription factor Crz1p, causing its translocation to the nucleus, where it's responsible for the expression of genes that induce tolerance to ethanol[2].

Other main objective was to investigate if growing both species in the presence of ethanol had some influence in the following increase of cytosolic Ca^{2+} concentration, in response to an ethanol shock. For *S. bayanus*, growth with 0, 3 or 9% ethanol leads to similar patterns, probably because the strain used in this study was naturally more resistant to ethanol. The results for *S. cerevisiae* neotype strain show that cells grown with 3% ethanol seem to respond in a more intense way to the ethanol shock, than cells grown without ethanol.

The experiments with *S. cerevisiae* BY wild type and deletion strains showed that ethanol wasn't acting through glucose-sensing or pheromone signaling GPCRs, but through an alternative pathway, to promote an increase of cytosolic Ca^{2+} concentration in yeasts.

Keywords: Ethanol; Calcium; GPCR; Stress response; *Saccharomyces* sp.

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1. Introduction

Yeasts are used in several industrial processes, such as the production of beer, wine and biofuel ethanol[9], as well as in science[10]. The most common yeast used in these processes is *Saccharomyces cerevisiae*[9] that, together with *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. mikatae*, *S. cariocanus* and *S. kudriavzevii*, belongs to *Saccharomyces sensu stricto* complex. This study will focus in *S. cerevisiae* and *S. bayanus*, which are the most distantly related species in this complex[11], with several chromosomes rearranged in a different manner, and capable of generating only sterile hybrids[12]. Besides that, they have in common most of the morphological traits and physiological properties, which was the reason for them being considered as belonging to the same species in the past[13]. In 2001, crosses between the two species generated fertile hybrids[14], setting again the controversy around the relatedness of *S. cerevisiae* and *S. bayanus*.

Both species are used in wine and beer making[15], but *S. bayanus* is also used in the production of cider and champagne, having the ability of growing in lower temperatures than *S. cerevisiae*, but not above 37º C[11]. Both are Crabtree-positive, which means that they are sensitive to small concentrations of glucose in the medium, shifting their metabolism from respiration to fermentation, even in aerobic conditions[15]. Fermentation results in the production of ethanol[10], that sometimes reach concentrations above 15% (v/v), which could be a major source of stress, affecting cell growth and viability, as well as fermentation efficiency. *S. cerevisiae* usually has a higher ethanol tolerance than *S. bayanus*, but the neotype strain of *S. cerevisiae* (used in the present study) is only capable to develop colonies in plates with 5% ethanol, but not with 10% ethanol. Other strains of the same species and of *S. bayanus* are capable of growing in the presence of 10% ethanol, and in some cases even above that concentration[16]. The strain of *S. bayanus* used in the present study was selected by its enhanced oenological properties, so it has a high resistance to ethanol (over 15% (v/v))[17].

Because 80% of world ethanol production is achieved by anaerobic fermentation by *S. cerevisiae*, and with the increasing demand for bioethanol fuel as a renewable and sustainable energy source[18], having strains resistant to ethanol is extremely important[19]. To achieve that, it's important to improve the knowledge on how ethanol influences yeast cells and, more specifically, how yeasts respond to this type of stress.

Ethanol is an amphipathic compound, capable of insertion into the plasma membrane hydrophobic interior, weakening this barrier and affecting the positioning and function of proteins, which leads to higher fluidity, permeabilization and leakage of amino acids and some cellular components, interfering with proton gradient and nutrient transport[20]. It can also inhibit the activity of some glycolytic enzymes and damage mitochondrial DNA[21].

Ethanol endogenously generated by yeast is more cytotoxic than exogenous ethanol in the same concentration[22].

Ethanol tolerance in *S. cerevisiae* appears to be related with sterol and phospholipid fatty acid composition of their plasma membranes, because this is the first sensitive structure that contacts with ethanol from the medium[23]. Namely, a higher content of monounsaturated fatty acids (oleic acid) and ergosterol in plasma membrane is a common response of *S. cerevisiae* to high ethanol concentration, that helps stabilize the membrane and avoid fluidity. There are also evidences that *S. cerevisiae* changes its gene expression, increasing expression of chaperone proteins (heat shock proteins) and trehalose in order to stabilize and/or repair denatured proteins[24]. A large number of genes can be up or down-regulated in response to this stress (including genes involved in energy metabolism, protein trafficking and biosynthesis, transport mechanisms, cell cycle and growth, ionic homeostasis, membrane and cell wall organization, and lipid and nucleotide metabolism), in order to restore normal cellular functioning[24,25]. There are reports that plasma membrane H^+-ATPase and vacuolar V-ATPase increases its activity, in order to counteract ethanol-induced proton influx from extracellular medium and corresponding intracellular acidification[21]. With respect to mitochondrial damage induced by ethanol, the production of oxygen free radicals is counteracted by increased activity of the antioxidant enzyme mitochondrial superoxide dismutase[22]. A study showed that addition of Ca^{2+}, as $CaCl_2$, to fermentation medium has a positive effect on ethanol tolerance of *S. cerevisiae* and *S. bayanus*, because it improves membrane stability[26]. Magnesium has also been shown has having protective effects against 10% (v/v) ethanol in fermentation medium[22]. Also the incorporation of some amino acids, as isoleucine, methionine, phenylalanine and proline, could counteract the fluidizing effect of ethanol[24].

When yeast are pre-exposed to a sublethal amount of a stressing agent like ethanol, it can activate stress response mechanisms that results in a transient resistance to higher levels of ethanol[1]. Also important is the fact that cellular responses to heat and ethanol shock show some overlap, which is evident in the fact that a sublethal heat shock induces temperature as well as ethanol tolerance in yeast, indicating the existence of cross-protection[22].

It is clear that many systems are involved in *S. cerevisiae* adaptation and tolerance to ethanol, since more than 200 genes appear to be involved, because their disruption leads to ethanol sensitivity[2]. The *S. cerevisiae* stress response to ethanol is associated with general stress response mechanisms, but there are some ethanol-specific responses that have been identified[1]. Recently, the alcohol-sensitive ring/PHD finger 1 protein (Asr1p) was identified as possibly being related to yeast ethanol tolerance, but more studies are necessary. When

yeast cells are exposed to ethanol stress, this protein exits the cytoplasm and accumulates in the nucleus, possibly controlling chromatin structure and transcription[24].

Other yeast stress response includes binding of Msn2p and Msn4p transcription factors to STRE- (stress response element) containing promoters, to stimulate expression of downstream genes. The accumulation of these proteins in the nucleus depends on one of several signalling pathways, as cAMP-protein kinase (PKA), TOR and HOG pathways[24]. Respecting to the first pathway, under ethanol stress, proteins of the Hsp70 family are recruited to assist in the refolding of ethanol denatured proteins, reducing their normal interaction with Cdc25p. It prevents this last protein from activating Ras1p/2p, leading to a decreasing in the generation of cAMP from adenylate cyclase. In this situation, the down-regulation of the cAMP-PKA pathway causes Msn2p/Msn4p transference from cytosol to the nucleus, to trigger the stress response[25].

Ethanol also activates the transcription factor Hsf1p, which induces expression of genes with heat shock elements (HSEs) in their promoters, including heat shock proteins. This type of stress can also activate the protein kinase C (PKC) cell integrity pathway, by activation of Slt2p, that is a mitogen-activated protein kinase (MAPK), resulting in higher ethanol tolerance[2]. There are evidences that this pathway has functional redundancy with the calcineurin pathway, acting together to maintain cell integrity and survival, in response to high temperatures, high NaCl concentrations or mating pheromone (α-factor)[2,27].

But, until recently, the participation of the calcineurin pathway in the ethanol stress response wasn't demonstrated. Calcineurin is a conserved Ca^{2+} and calmodulin dependent phosphatase. In response to a stress, cytosolic Ca^{2+} levels rise, resulting in the formation of Ca^{2+}/calmodulin complexes that activate calcineurin. When activated, calcineurin dephosphorylates the transcription factor Crz1p, causing its rapid translocation to the nucleus[2,28]. Crz1p is responsible for the expression of stress responsive genes under the control of a CDRE (calcineurin-dependent response element). These genes are involved in signalling pathways, ion/small molecule transport, cell wall maintenance, and vesicular transport[29]. Recently, it was demonstrated the involvement of the calcineurin/Crz1p pathway in ethanol stress adaptive tolerance[2]. It was demonstrated that ethanol stimulates calcineurin-dependent nuclear localization of Crz1p, that this transcription factor induces CDRE-dependent gene expression and that this pathway is responsible for inducing adaptive tolerance to higher ethanol concentrations, when cells are pre-treated with 8% (v/v) ethanol[2]. But, it remains to be proved if there is an increase in cytosolic Ca^{2+} concentration after ethanol shock, and if so, from where does it originates? One study with pancreatic acinar cells showed that ethanol was responsible for a transient increase in cellular Ca^{2+} levels, and this Ca^{2+} was released from the endoplasmic reticulum[30].

For Ca^{2+} to act as a second messenger in signal transduction, its concentration has to increase transiently in the cytosol, triggered by a stimulus. For that to happen, cytosolic free Ca^{2+} concentrations have to be actively maintained at extremely low levels, in spite of the existence of steep Ca^{2+} gradients across the plasma membrane or intracellular membranes. The possible sources of Ca^{2+} are the extracellular medium or intracellular stores. The vacuole is the major intracellular non-exchangeable Ca^{2+} pool, where yeasts accumulate more than 90% of the Ca^{2+}, functioning like a store and a sink. The smaller pool is highly exchangeable with external Ca^{2+}, being in the cytosol or in secretory organelles[31].

Several channels, antiporters and pumps are responsible for Ca^{2+} transport between different compartments and the extracellular medium, which helps to maintain an intracellular steady-state of Ca^{2+} concentration of about 0.1 μM[31]. These channels and pumps are homologous to those involved in Ca^{2+} homeostasis in mammalian cells[32]. In the yeast vacuolar membrane exists a H^+/Ca^{2+} antiport (Vcx1p) that transports Ca^{2+} into the vacuole, and is dependent on the transmembrane pH gradient normally produced by the vacuolar H^+ V-ATPase[33]. The same membrane also contains Pmc1p, which is a high-affinity Ca^{2+} pump that transports Ca^{2+} into the vacuole. The Golgi complex or related secretory compartments (endoplasmatic reticulum) also has a Ca^{2+} pump (putative Ca^{2+}-ATPase), encoded by the *PMR1* gene. Its function is to supply the Golgi with Ca^{2+} necessary for specific secretory functions, maintaining at the same time the homeostasis of cytosolic free Ca^{2+} concentration. Yeast mitochondria accumulate little Ca^{2+} and its role in Ca^{2+} metabolism is poorly understood[31].

Increase in cytosolic Ca^{2+} in response to different stresses could be from Ca^{2+} entry across plasma membrane where is a high affinity Ca^{2+} influx channel, constituted by the subunits Cch1p and Mid1p[34], that are probably regulated by calcineurin[35]. There is some evidence that extracellular Ca^{2+} could also enter the cytosol through an unknown channel X, whose encoded gene has not been identified yet[36]. Under some conditions, Ca^{2+} could also be released from the vacuole via Yvc1p, which is a voltage-dependent Ca^{2+}-activated channel in vacuolar membrane[28,37].

One of the reasons for yeasts to increase their cytosolic Ca^{2+} concentration is the activation of GPCRs (G-protein coupled receptors), through binding of an agonist molecule outside the cell. Binding of the ligand results in a change in the receptor protein conformation, which then activates the coupled G-protein[38].

Besides agonist molecules, that produce a functional response (activation) after binding to the receptor, there are molecules that work as neutral antagonists or inverse agonists. The antagonists block the effect of an agonist, and could be one of three types: competitive, non-competitive[39] and uncompetitive[40]. In the competitive case, both agonist and antagonist bind to the same site on the receptor, so the action of the antagonist could be overcomed using

higher concentrations of the agonist. In the non-competitive type, the binding sites for agonist and antagonist are different, so increasing doses of agonist will not overcome the antagonist effect, because agonist can't displace the antagonist molecule[39]. An uncompetitive antagonist is when its action is contingent upon prior activation of the receptor by the agonist, so the same concentration of antagonist blocks better higher concentrations of agonist, than lower concentrations. The case of inverse agonists happens when the receptor presents constitutive activity, being active even in the absence of an agonist. An inverse agonist will inhibit this constitutive activity[40], and this is the opposite of what happens with a neutral antagonist, which has no activity in the absence of the agonist[41].

Although human cells contain hundreds of types of GPCRs, in *S. cerevisiae* only two distinct GPCR systems have been identified so far: one for glucose-sensing and other for pheromone signalling. Although most eukaryotic organisms have GPCRs capable of mediating responses to a huge variety of extracellular signals, a nutrient like glucose isn't a common stimulus for this type of receptors[6].

The glucose-sensing GPCR system is constituted by the glucose receptor Gpr1p and the Gα protein (Gpa2p), but no β or γ subunits have been identified yet. Gpa2p is thought to activate adenylate cyclase, which leads to cAMP synthesis, activating cAMP-dependent protein kinase (PKA) that is responsible for controlling several targets. This response results in the switch from respiration to fermentative metabolism. Agonists for this GPCR are D-glucose and sucrose, while mannose is the antagonist[6] (and not an inverse agonist, because it doesn't have an inhibitory effect on basal cAMP level). The same GPCR system has been implicated in glucose-induced Ca^{2+} signalling, through phospholipase C (Plc1p) stimulation[42]. More specifically, Gpr1p physically interacts with Plc1p, and there are evidences that activated Plc1p leads to phosphatidylinositol 4,5-bisphosphate (PIP_2) turnover in diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3)[43]. The IP_3 generation seems to be sufficient for raising the calcium level in the cytosol[44], but the downstream pathway isn't fully known yet. Namely, in mammalian cells, IP_3 causes Ca^{2+} release from intracellular stores, activating protein kinase C (that is also activated by diacylglycerol directly), but in yeasts hasn't been proved yet the direct link between Plc1p activation and protein kinase C activation, and the increase of cytosolic Ca^{2+} concentration seems mainly due to an influx from external medium[43].

In respect of the GPCR involved in pheromone signalling, when pheromones (α or a-factor) bind to the respective receptor (Ste2p[38] or Ste3p[45], respectively), it stimulates the exchange of GDP for GTP on the Gα protein (Gpa1p), which in turn dissociates from the βγ dimer (Ste4p and Ste18p). This βγ dimer transmits the signal to the protein kinase Ste20p, activating a MAP-kinase cascade. The final objective of this response is the fusion with a yeast cell of the opposite mating type[6]. Pheromones are also responsible for the increase in

levels of intracellular Ca^{2+}, which induces activation of the calmodulin-dependent kinases and calcineurin that in turn influences the spatial localization of Crz1p, as explained above. The source of this Ca^{2+} seems to be the extracellular medium[46,47].

Eukaryotic GPCRs, in general, are able to detect and mediate rapid responses to extracellular signals. Around 30-40% of all clinically prescribed drugs function as GPCR agonists or antagonists, and most of the human GPCRs are orphan, which means that their ligand hasn't been found so far. So, this is a very promising area to study, because these orphan receptors can be targets for the development of new drugs. Due to the similarity to GPCR-mediated signalling in humans, the pheromone signalling GPCR of *S. cerevisiae* can serve as a model and screening tool of agonists and antagonists, for the study of GPCR systems in higher eukaryotic cell types. To do that, the yeast pheromone signalling pathway is coupled with mammalian receptors heterologously expressed in *S. cerevisiae* cells[6]. Basically, the yeast pheromone receptor has to be replaced by the mammalian GPCR, and the G-protein has to be tailored in order to couple the new GPCR to the pheromone pathway. Finally, the output of this pathway has to be engineered, in order to enable a fast detection of the activation by the possible ligands[7]. The main advantages of using yeasts to express and study heterologous GPCRs are related to the low cost of growth and maintenance, and also with the facility and flexibility to manipulate by genetic and molecular procedures[48]. They are also ideal for high-throughput screenings, because of their high resiliance. Furthermore, it's easy to eliminate all endogenous GPCR and Gα subunits in *S. cerevisiae*, in order to have clear and unambiguous results from the expression of the heterologous GPCRs[8]. If the present study proves that ethanol stress response acts through a GPCR, it can be used as an experimental control situation to test high-throughput microchips based on fluorescence variation to detect GPCR activation. Furthermore, in future studies, it will be also interesting to express mammalian orphan GPCRs in yeasts, in order to screen libraries of putative ligands.

The first objective of this study is to verify if there is an increase in cytosolic Ca^{2+} concentration after the ethanol shock, in *S. cerevisiae* and *S. bayanus*, and if so, what is the provenience of that Ca^{2+}. Another goal will be to analyse if the growth in the presence of ethanol, which presumably will activate the stress response mechanisms, lead to a different pattern of Ca^{2+} response to an ethanol shock. Because pheromone signalling GPCR can act by calcineurin/Crz1p pathway, and ethanol also acts by this pathway, other main goal of this study is to investigate if the increase in cytosolic Ca^{2+} concentration is due to the activation of a GPCR by ethanol. Since most of the studies are centered on *S. cerevisiae*, it will be interesting to analyse the differences between this species and *S. bayanus*.

2. Materials and methods

2.1. Yeast strains

Saccharomyces species used in the present study were *S. bayanus* and *S. cerevisiae* (strains listed in Table 1).

Table 1. *S. bayanus* and *S. cerevisiae* strains studied, their relevant genotypic information and source.

Strain code	Strain name	Relevant genotypic information	Gene deleted	Source
	S. bayanus			Institut Oenologique de Champagne, Epernay, France
PYCC 4455	*S. cerevisiae* Meyen ex E.C. Hansen *var. cerevisiae* (CBS 1171, neotype of *S. cerevisiae*)			Portuguese Yeast Culture Collection (PYCC), New University of Lisbon, Portugal
Y00000	*S. cerevisiae* BY4741 (BY wild type reference strain)	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0		Euroscarf (EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis), Frankfurt, Germany
Y03731	*S. cerevisiae* BY4741 (deletion strain)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YDL035c::kanMX4	GPR1 (YDL035c)	Euroscarf, Frankfurt, Germany
Y05645	*S. cerevisiae* BY4741 (deletion strain)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YFL026w::kanMX4	STE2 (YFL026w)	Euroscarf, Frankfurt, Germany
Y04847	*S. cerevisiae* BY4741 (deletion strain)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YGR217w::kanMX4	CCH1 (YGR217w)	Euroscarf, Frankfurt, Germany
Y01153	*S. cerevisiae* BY4741 (deletion strain)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YNL291c::kanMX4	MID1 (YNL291c)	Euroscarf, Frankfurt, Germany
Y01863	*S. cerevisiae* BY4741 (deletion strain)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YOR087w::kanMX4	YVC1 (YOR087w)	Euroscarf, Frankfurt, Germany

2.2. Yeast growth media and conditions

Cells were grown in 20 mL of YEPG liquid medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, distilled water) with pH 5.5, at 30ºC, with orbital agitation (250 rpm). The initial OD_{600nm} was 0.1, and cells were grown until they reached late exponential/early stationary phase, which was followed for each species and growth condition.

In the conditions of growth in the presence of ethanol, it was added to the media until it reached a final concentration of 3% or 9% ethanol (v/v), maintaining the final volume of 20 mL.

Cell banks stored at -80ºC were made for all species, using 20% glycerol. Working stocks were grown in YEPG solid medium with 2% (w/v) agar, at 30ºC, and stored at 4ºC.

2.3. Fluo-4 AM

Fluo-4 belongs to a new group of synthetic fluorescent indicators with visible excitation and emission wavelengths (exc=494 nm; em=516 nm)[49], which allows measurements and quantification of cytosolic free Ca^{2+}, in the 100 nM to 1 µM range[3]. All Ca^{2+} indicators, including Fluo-4, are membrane impermeable in their Ca^{2+}-sensitive form (salt form). The method found to introduce indicators into cells was to introduce an AM (acetoxymethyl)-ester linkage in their structure, which makes Fluo-4 almost nonfluorescent[4]. In this work, it was used the Fluo-4 AM reagent contained in Fluo-4 Direct Ca^{2+} Assay Kit, from Invitrogen. But, probably due to the existence of cell walls in yeasts, it doesn't diffuse passively through them, unlike animal cells. For that reason, it was necessary to use an electroporation protocol, adapted from one existing for Fura-2 fluorophore[50].

Once inside the cell, the AM-ester groups are cleaved by endogenous esterases, releasing the Ca^{2+}-sensitive form intracellularly, that are unable to leave cells by diffusion. But, at least for animal cells, anion transporters present in the plasma membrane can cause the leakage of indicators to extracellular medium[4]. For that reason, it was added probenecid to Fluo-4 AM reagent, which is an organic anion-transport inhibitor, reducing leakage of the de-esterified indicator. Probenecid also reduces the baseline signal[51].

Basically, Fluo-4 AM suffers an increase of fluorescence intensity induced by free Ca^{2+} binding. This is possible because this type of indicators possess a BAPTA-like Ca^{2+}-chelator component covalently linked to a fluorogenic, fluorescein-like component[3]. Fluo-4 is an analog of fluo-3, but with the two chlorine substituents replaced by fluorines[51].

Fluo-4 tends to show a uniform cytoplasmic fluorescence, but since these types of indicators could also cross other cellular membranes, mitochondrial/ER staining was seen in a minority of animal cells. This fluorescent indicator is also very resistant to photobleaching, which consists in the irreversible damage of Ca^{2+}-indicator molecules, leading to a decrease of fluorescence signal over time. It also has a large dynamic range, providing images with high contrast[4]. Its Ca^{2+} affinity is around K_d =345 nM, and has low background absorbance[3].

Typically, fluorescent Ca^{2+} indicators are widely used for in-cell measurement of agonist-stimulated and antagonist-inhibited Ca^{2+} signalling through G protein-coupled receptors (GPCRs).

The Fluo-4 AM reagent was ressuspended with 10 mL of zero-Ca^{2+} buffer (with HEPES 1 M and Hanks Buffered Salt Solution 1x, without $CaCl_2$ and $MgCl_2$, pH 7.3[52]) and 200 µl of probenecid stock solution (also prepared with zero-Ca^{2+} buffer). In the end, the final concentration of probenecid is 5 mM and the Fluo-4 AM reagent is 2x concentrated. The exact concentration of Fluo-4 AM wasn´t provided by Invitrogen.

2.4. Microplate fluorescence assays

Freshly grown cells, in late exponential/early stationary phase (determined by OD_{600nm} measurement), were used to perform the microplate fluorescence assays. To ensure that the results are comparable between different assays, the cell number was calculated with a hemacytometer. The necessary volume was collected, in order to have the same number of cells in the different growth conditions (0, 3 or 9% ethanol in the growth medium) and for the different strains. Yeast cells were then washed 3 times with 1 mL of milli-Q water, by centrifugation (3800 g, 2 minutes), to remove any traces of medium. Cell pellets with the same cell number (3 x 10^8 cells/mL[53]) were ressuspended with 100 µl of the Fluo-4 AM reagent solution in the appropriate concentration, depending on the species and growth condition. This means that, for *S. bayanus*, the Fluo-4 AM dilution factor used was 1:2, which corresponds to the dilution suggested in the Invitrogen protocol. For *S. cerevisiae* neotype strain, the Fluo-4 AM dilution factor used was 1:8 for cells grown with 0% ethanol, and was 1:32 for cells grown with 3% ethanol. For *S. cerevisiae* BY wild type and deletion strains, all grown in the absence of ethanol, the Fluo-4 AM dilution factor used was 1:2.

The cells ressuspended with the Fluo-4 AM reagent solution were transferred to 2 mm electroporation cuvettes. After 20 minutes in ice, the cells were submitted to electroporation in a BTX ECM399 electroporator, in order to Fluo-4 AM enters yeast cells. The voltage and number of milliseconds depended on the experiment conditions. Since the electroporator didn't allow to control the duration of each pulse, in terms of number of milliseconds, it was necessary to give more than one pulse until the pretended total number of milliseconds were achieved. Then, cells were again washed 3 times with 1 mL of milli-Q water to remove traces of Fluo-4 AM reagent from the exterior of the cells, and ressuspended in the zero-Ca^{2+} buffer[52].

Depending on the experiments, different solutions (ethanol, $CaCl_2$, glucose, mannose and EDTA) were added to the respective wells of a 96 well white microplate (flat bottom, no treatment, Corning Science). All the solutions were prepared in milli-Q water, in a higher concentration, so that after addition of the cells and other solutions, they all have the correct final concentration. Then, yeast cells were added to the same wells, in order to always have the same volume of cells (100 µl), and the same cell number (2.4 x 10^7 cells). In the end, all the wells had a final volume of 200 µl. The fluorescence intensity was measured immediately

after the cells addition, using a Varian Cary Eclipse Fluorescence Spectrophotometer (Advanced Reads software). The excitation and emission wavelengths were 494 nm and 516 nm, respectively, the excitation and emission slits were 5 nm, and the photo multiplier gain was set on high mode. The fluorescence intensity value in each well is the average value of 5 replicate measurements performed automatically by the instrument, in the center of the well.

Negative control conditions consisted in yeast cells that were submitted to the same treatment, except that in the electroporation phase, it was added zero-Ca^{2+} buffer in the correspondent dilutions, instead of Fluo-4 AM reagent. In that way, these cells will only have autofluorescence. This value of autofluorescence, for which the cells and also the microplate contributed, was then subtracted from the results.

The electroporation protocol used was adapted from one existing for Fura-2 fluorophore[50].

2.5. Flow cytometry assays

Flow cytometry assays were always performed after the microplate fluorescence assays. First, cells were collected from the necessary wells and diluted in Dulbecco's Phosphate-Buffered Saline[54] (PBS, pH 7.2), to obtain around 10^6 cells. After centrifugation (9900 g, 2 minutes), to remove traces of previous solutions, cells were ressuspended in 100 µl of PBS, and transferred to 5 mL polystyrene round-bottom tubes (Falcon). Then, 5 µl of propidium iodide (Biolegend) were added to each tube, incubating for 15 minutes at dark. Cells were washed with 2 mL of PBS, by centrifugation, to remove excess of propidium iodide. In the end, they were ressuspended in 500 µl of PBS, and measured in a Becton Dickinson FACSCalibur flow cytometer, that analyzed 10,000 cells in all conditions.

The cell population of interest was gatted using a scatter plot with information about forward scatter and side scatter. That cell population was then divided, respecting their fluorescence intensity in green (Fluo-4 AM; FL1) and red (propidium iodide; FL3) wavelengths. The quadrants resulting from that give information about the number of cells that are alive and injured/dead (percentage of cells excluding or not the propidium iodide, respectively), and at the same time, give information about presence or absence of green fluorescence from Fluo-4 AM in the cells. The program used to analyze the results was Cell Quest Pro. The quadrants were adjusted using information about the distribution of cells without any fluorescence, and with only one of each stains. To validate the results, it were also tested some control samples with 100% heat-killed and 100% live yeast cells.

2.6. Control assays with cutinase enzyme

In order to exclude the hypothesis that ethanol could be interacting unspecifically with Fluo-4 AM, a control assay was designed. Fluo-4 is in the form of an acetoxymethyl ester,

and the group acetoxymethyl has to be cleaved by intracellular esterases in order to Fluo-4 become fluorescent. Since cutinase is an esterase, it was used to make that cleavage in the absence of cells.

An equal volume of Fluo-4 AM reagent and cutinase enzyme (1 mg/mL, from *S. cerevisiae*, diluted in phosphate buffer, 20 mM, pH 7.0) was mixed, and incubated overnight at 37ºC. In a control condition, instead of cutinase, only phosphate buffer was added to Fluo-4 AM. Then, 10 µl of this mixture were added to ethanol solutions in different concentrations, with or without Ca^{2+}, with a final volume of 210 µl. Ca^{2+} solutions were from Ca^{2+} Calibration Buffer Kit #1 (Invitrogen), consisting in a zero free-Ca^{2+} buffer (10 mM K_2EGTA) and a 39 µM free-Ca^{2+} buffer (10 mM CaEGTA). The fluorescence intensity was measured in 96-wells white microplates (exc=494 nm; em=516 nm).

2.7. Fluo-4 AM spectra determination and characterization

In order to obtain excitation and emission spectra of Fluo-4 AM, it was used Ca^{2+} Calibration Buffer Kit #1 (Invitrogen). Again, an equal volume of Fluo-4 AM reagent and cutinase enzyme (1 mg/mL, from *S. cerevisiae*, diluted in phosphate buffer, 20 mM, pH 7.0) was mixed, and incubated overnight at 37ºC. Then, 50 µl of this mix were added to 1 mL of zero Ca^{2+} solution, and 150 µl to 3 mL of 39 µM Ca^{2+} solution. The zero and 39 µM Ca^{2+} solutions with cleaved Fluo-4 AM reagent were cross-diluted to produce a series of 11 solutions with an increasing amount of total free Ca^{2+}. The fluorescence intensity of each dilution was measured in 1 mL cuvettes (exc=494 nm; em=516 nm).

2.8. Determination of intracellular Ca^{2+} concentration

Using solutions from the Ca^{2+} Calibration Buffer Kit, it was possible to determine the relation between Ca^{2+} concentration and fluorescence intensity values. *S. bayanus* or *S. cerevisiae* neotype strain cells ressuspended in the zero-Ca^{2+} buffer (100 µl) were used to spike solutions with different Ca^{2+} concentrations and 2.5 µM of Fluo-4 salt. The fluorescence intensity values were determined in a total volume of 200 µl, using 96-wells white microplates (exc=494 nm; em=516 nm).

2.9. Statistical analysis

After autofluorescence subtraction and determination of fluorescence variation values (when applicable), averages and ±standard errors of the mean (SEM) were calculated (when biological replicates were performed) using Microsoft Office Excel 2007 software. Simple linear regression analysis was applied to some results, using the same software. The linearity of the regressions was assessed using Analysis of Variance (ANOVA) linearity test (F-test), performed in SPSS Statistics 17.0. The significance of the linear response was first

tested looking at the linearity test results (null hypothesis is that the response is non-linear). When these results were significant (p-value<0.05), deviation from linearity was also tested (null hypothesis is that the response is linear). When only one biological replicate of the results was obtained, the linearity of the response wasn't tested, because SPSS software requires multiple observations of Y. In these cases, the R^2 values were used as indicative of the proportion of variability that is accounted for by the statistical model.

It was also tested if the slopes were significantly different from zero (null hypothesis is that the overall slope is zero), using a linear regression ANOVA (F-test), performed in GraphPad Prism 5.04. When the linearity of the response wasn't confirmed, the analysis finished at this point, and the discussion of the results had to be based on the graphic representations. The statistical significance of these interpretations couldn't be assessed and the lines in the figures just illustrate the general trends.

When the linearity of the response was confirmed, the slopes (and elevations, when applicable) of two or more data sets were tested to determine if were significantly different (null hypothesis is that the slopes are equal), using the same software. It compares linear regressions using a method equivalent to Analysis of Covariance (ANCOVA)[55]. Results were considered significant if p-value<0.05.

3. Results and discussion

3.1. Fluo-4 AM spectra determination and characterization

In order to characterize Fluo-4 AM and confirm the optimum excitation and emission wavelengths to measure the fluorescence emitted by this fluorophore, the excitation and emission spectra were determined (Fig. 1).

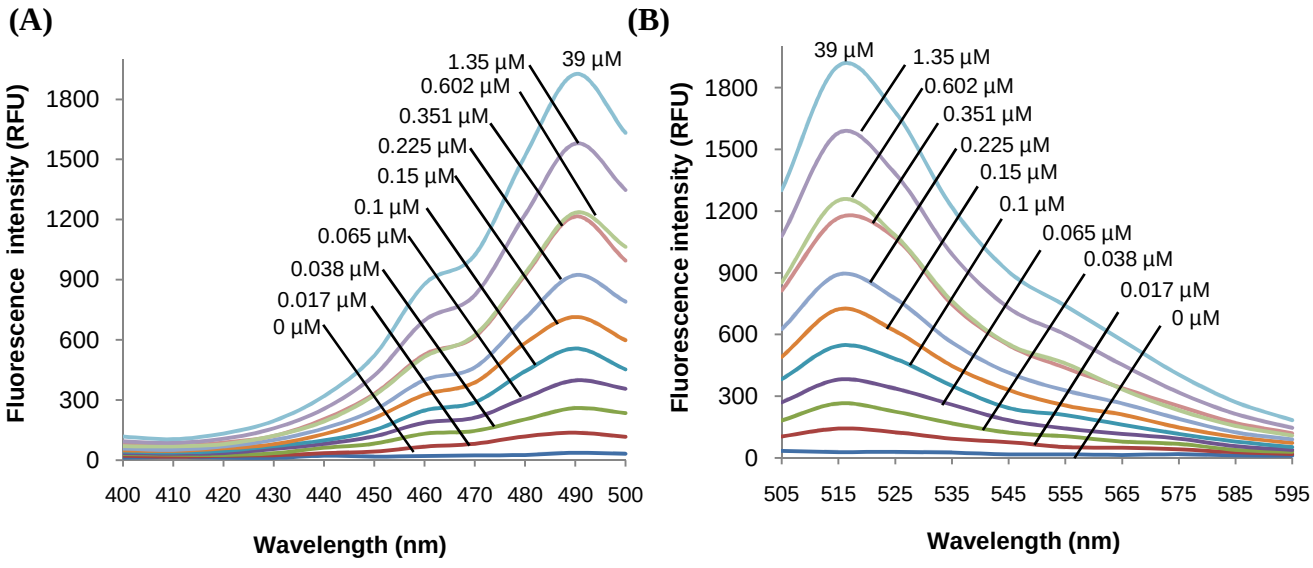


Fig. 1. Fluorescence spectra of Fluo-4 AM.
(A) Fluorescence excitation (fixed emission=516 nm) and (B) emission (fixed excitation=494 nm) spectra of Fluo-4 AM, as a function of increasing Ca^{2+} concentrations.

Both results were accordingly with expected spectra, with fluorescence intensities increasing with successively higher free Ca^{2+} concentrations, and with the peaks in the expected wavelengths (excitation = 494 nm; emission = 516 nm). These results indicate that the method used to cleave Fluo-4 AM *in vitro* was successful, and the detection parameters of the fluorescence spectrophotometer were adequate.

3.2. *Saccharomyces bayanus* and *S. cerevisiae* neotype strain (PYCC 4455) characterization

The growth curves for *S. bayanus* (Fig. 2A) and *S. cerevisiae* neotype strain (Fig. 2B), with 0%, 3% or 9% ethanol in growth medium, were determined, in order to ensure that all experiments were performed with yeast cells in the same growth phase. The desired phase was the late exponential/early stationary, which was achieved by *S. bayanus* at 16h, 21h and 41h, growing in the presence of 0, 3 or 9% ethanol (v/v), respectively. To reach the same phase, *S. cerevisiae* neotype strain took 20h and 25h, in the conditions with 0 and 3% ethanol in the growth medium.

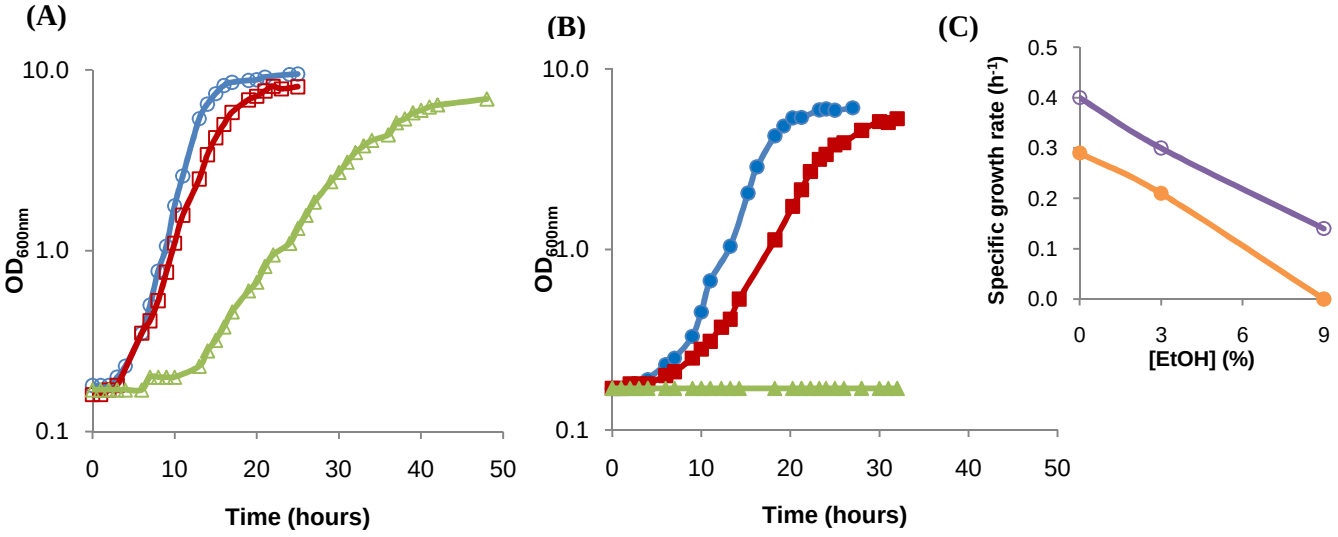


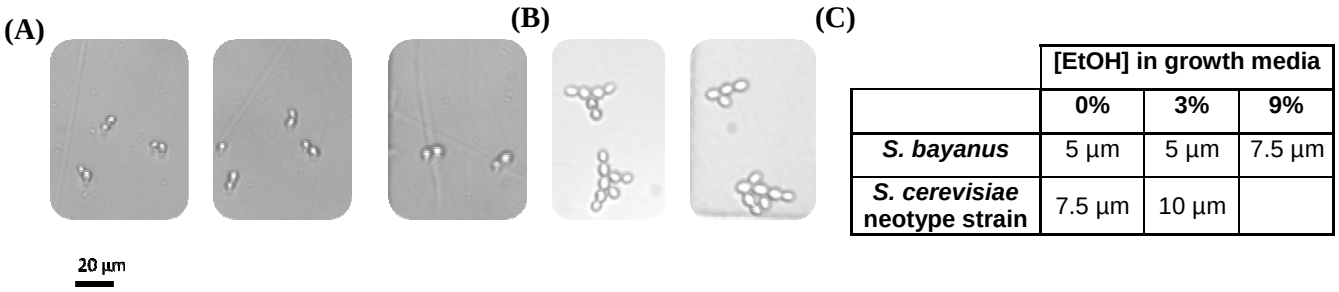
Fig. 2. *S. bayanus* and *S. cerevisiae* neotype strain growth curves.
(A) *S. bayanus* (open symbols) and (B) *S. cerevisiae* neotype strain (closed symbols) growth curves, with 0% (○, ●), 3% (□, ■) or 9% ethanol (△, ▲) (v/v) in the growth medium. Logarithmic scale was applied to yy axis. (C) Specific growth rates were 0.4, 0.3 and 0.14 h^{-1} for *S. bayanus* (○) growing with 0, 3 or 9% ethanol (v/v), respectively, and 0.29, 0.21 and 0 h^{-1}, for *S. cerevisiae* (●) growing with 0, 3 or 9% ethanol (v/v), respectively. Cells were grown in YEPG liquid medium, pH 5.5, 30ºC, 250 rpm, with an initial OD_{600nm} of 0.1.

The specific growth rates of both species grown with different ethanol concentrations will also give information about their resistance and adaptability to ethanol. As expected, in both species, specific growth rates decreased (Fig. 2C), with increasing ethanol concentration in the growth medium. This impairment is due to the fact that ethanol, in low concentrations, affects cell growth, and in high concentrations could affect viability[16]. Mainly, ethanol weakens the plasma membrane, increasing its fluidity and permeability, interfering with

proton gradient and nutrient transport, which increases the metabolic burden[20]. So, yeast cells subjected to ethanol stress would take some time to adapt to those ethanol concentrations, triggering general stress response mechanisms, as well some ethanol-specific responses that already have been identified[1,2,24,25]. This was well illustrated by the longer lag phase of *S. bayanus* grown with 9% ethanol, when compared with the remaining growth conditions.

S. cerevisiae neotype strain was unable to grow in a medium containing 9% ethanol (v/v), unlike *S. bayanus*, and for the same ethanol concentrations, *S. cerevisiae* has shown always lower specific growth rates (Fig. 2C). This is consistent with the results of a previous study, in which the neotype strain of *S. cerevisiae* (used in the present study) was only capable to develop colonies in plates with 5% ethanol, but not with 10% ethanol[16]. Other strains of the same species and of *S. bayanus*, were capable of growing in the presence of 10% ethanol, and in some cases even above that concentration[16]. It's important to refer that the strain of *S. bayanus* used in the present study was selected by their enhanced oenological properties, like the high resistance to ethanol (over 15% (v/v))[17], which also helps to explain the differences observed between the two species.

Yeast's morphology and dimension were also registered, in order to better characterize both species (Fig. 3).



	[EtOH] in growth media		
	0%	**3%**	**9%**
S. bayanus	5 µm	5 µm	7.5 µm
***S. cerevisiae* neotype strain**	7.5 µm	10 µm	

Fig. 3. *S. bayanus* and *S. cerevisiae* neotype strain morphology and diameter.
(A) *S. bayanus* cells after growing in medium with 0, 3 or 9% ethanol (v/v). (B) *S. cerevisiae* neotype strain cells after growing in medium with 0 or 3% ethanol (v/v). (C) Yeast's estimated diameter in the different growth conditions. Cells were grown in YEPG liquid medium, pH 5.5, 30ºC, 250 rpm, with an initial OD600nm of 0.1. Pictures were taken in a Leica DLMB microscope, with a Leica DC 350FX camera (200x magnification).

On average, *S. bayanus* cells had shown smaller diameters than *S. cerevisiae* neotype strain, which is consistent with data of current taxonomic studies[9]. In both species, the presence of ethanol in the growth medium appeared to be responsible for larger cell diameters. This can be explained by the fact that ethanol causes a cell-cycle delay, with a transient dispersion of F-actin cytoskeleton, which is responsible for a bigger cell size, and also for the slower growth rates[56,57].

Both species presented a globose or ovoidal morphology, as expected[9], and *S. cerevisiae* seemed to be more prone to aggregate in small groups of cells.

3.3. Fluo-4 AM protocol optimization

Some of the main objectives of this study were to verify if there was an increase in cytosolic Ca^{2+} concentration after an ethanol shock, in *S. cerevisiae* neotype strain and *S. bayanus*, and if so, what was the provenience of that Ca^{2+}. In order to detect the increase in cytosolic Ca^{2+} concentration, it was necessary to develop a way to deliver Fluo-4 AM inside yeast cells. Since it doesn't diffuse passively through yeasts, probably due to the existence of cell walls, an electroporation protocol was adapted from one existing for Fura-2 fluorophore[50].

This electroporation method isn't described in literature for Fluo-4, so it had to be optimized, to found the best conditions for loading this fluorescent indicator in these cells. The main aspects tested were the Fluo-4 AM concentration used, the voltage applied, as well as the total number of milliseconds that cells were subjected to electroporation. The objective was to found the best conditions in order to have a higher fluorescence in cells, but without killing too many of them.

3.3.1. *S. bayanus*

In order to optimize the protocol, *S. bayanus* cells were subjected to 10, 20, 25 or 30 ms of electroporation with 2500 V/cm, or 12 ms with 5000 V/cm. It was chosen a Fluo-4 AM dilution factor of 1:2, based on the Invitrogen protocol. After the washing steps, the cells were ressuspended in a Ca^{2+}-free buffer, which means that external Ca^{2+} was only available for the cells when 10 mM $CaCl_2$ were added. This concentration was chosen based on literature[43,58,59].

In a 96 well white microplate, the cells were subjected to an ethanol shock, using different ethanol concentrations (0, 2, 6, 8, 12 and 16% ethanol), and in the presence or absence of external Ca^{2+}. The fluorescence intensity was measured immediately after the ethanol shock, in a fluorescence spectrophotometer. This optimization procedure was only done for *S. bayanus* grown with 0% ethanol.

The fluorescence results will be referred in two different ways: fluorescence intensity and fluorescence variation values. The fluorescence intensity values correspond to the actual fluorescence value measured, after the autofluorescence value had been subtracted, and will be presented only in the appendix tables. In order to be possible to see more clearly the ethanol's effect in fluorescence and to facilitate the comparison between different conditions and species, the graphics will represent the fluorescence variation values. This means that to every fluorescence intensity value was subtracted the basal value (0% ethanol shock) of the same condition, in order to see how much the fluorescence increases between the basal condition (0% ethanol shock) and the conditions with increasing ethanol shock concentrations.

The results show that fluorescence intensity, as well as fluorescence variation between increasing ethanol shock concentrations and basal condition (0% ethanol shock for each electroporation condition) (Fig. 4; Appendix 1 for fluorescence intensity values), were higher with increasing number of milliseconds of electroporation, being the highest when using 5000 V/cm. The condition of 10 ms of electroporation was immediately discarded due to the low fluorescence intensity and variation presented. The fluorescence variation values also increased significantly in all conditions with increasing ethanol shock concentrations (all slopes were significantly different from zero, with $p<0.01$).

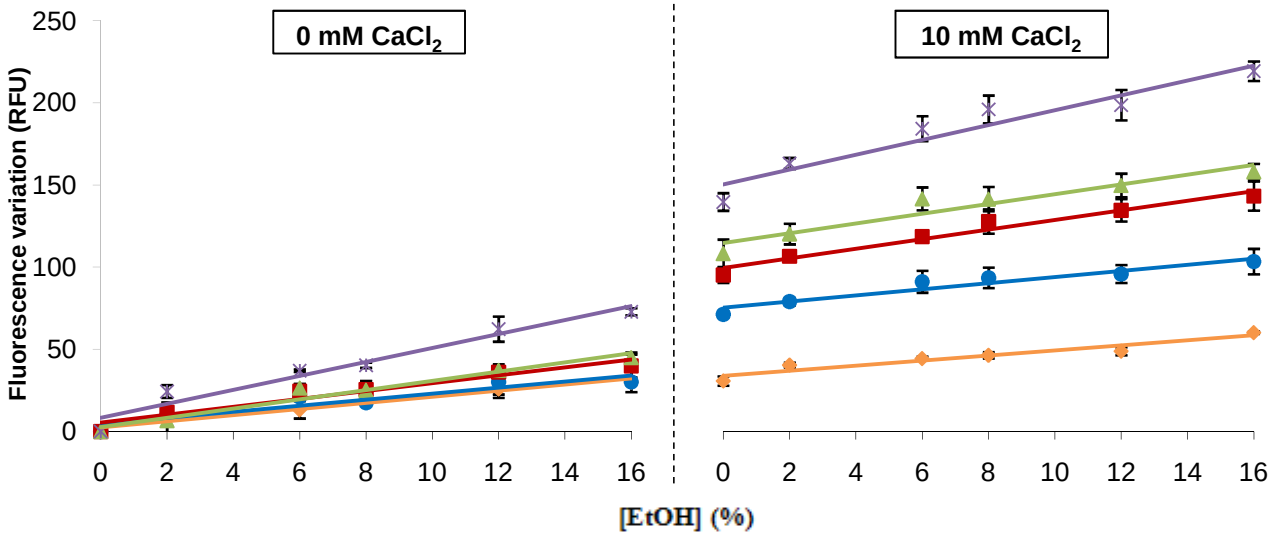


Fig. 4. Influence of the number of milliseconds of electroporation in *S. bayanus* Ca^{2+} response to ethanol shock. *S. bayanus* values of fluorescence variation, after 10 (♦), 20 (●), 25 (■) or 30 ms (▲) of electroporation with 2500 V/cm, or 12 ms with 5000 V/cm (×). The autofluorescence values were subtracted to all results, and presented as a function of increasing ethanol shock concentrations (v/v), with or without addition of 10 mM $CaCl_2$. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown. Linear response was confirmed for all conditions ($p<0.01$ in linearity test and $p>0.05$ in deviation from linearity test), and all slopes were significantly different from zero ($p<0.01$).
For 0 mM $CaCl_2$, the linear regression equations are $y = 1.8595x + 2.4749$; $y = 1.8287x + 4.7289$; $y = 2.404x + 5.3367$; $y = 2.8113x + 2.7942$; $y = 4.2445x + 8.3361$, for 10, 20, 25 and 30 ms with 2500 V/cm, and 12 ms with 5000 V/cm, respectively. For 10 mM $CaCl_2$, the linear regression equations are $y = 1.5446x + 33.808$; $y = 1.8741x + 75.258$; $y = 2.9198x + 99.584$; $y = 2.9724x + 114.7$; $y = 4.5077x + 150.4$ for 10, 20, 25 and 30 ms with 2500 V/cm, and 12 ms with 5000 V/cm, respectively.

But, the addition of 10 mM $CaCl_2$ didn't significantly altered the response, because for all electroporation conditions, the slopes weren't significantly different when $CaCl_2$ was added, when comparing with the 0 mM $CaCl_2$ situation ($p>0.05$). It's important to highlight that the fluorescence variation values, when $CaCl_2$ was added, had a high initial difference (0% ethanol shock condition). This is probably happening because longer electroporations create more pores in the cell membranes, leading to more Ca^{2+} entering in yeast cells. So, it's more correct to compare the slopes between the conditions with or without 10 mM $CaCl_2$, instead of the fluorescence variation values *per se*, to evaluate if external Ca^{2+} significantly alters the yeasts response. These results seem to indicate that cells were responding to ethanol shock with an increase of cytosolic Ca^{2+} concentration, and that response could occur in the absence of external Ca^{2+}.

In order to choose the best electroporation conditions, it was necessary to investigate its effect on cell viability and number of cells effectively fluorescent. To accomplish that, after the microplate fluorescence assays, the same cells were tested in a flow cytometer, but only for the conditions without addition of $CaCl_2$. The flow cytometer results (Fig. 5A) show that a higher number of milliseconds of electroporation leaded to an increasing loss of viability (statistical significance wasn't calculated due to lack of significantly linear response, in Fig. 5B).

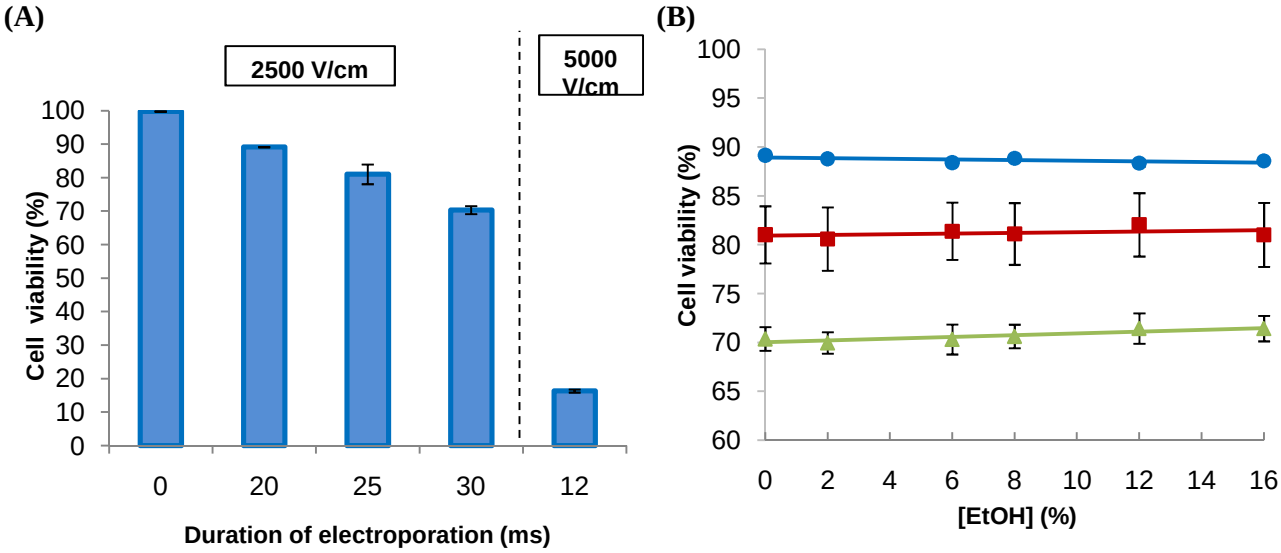


Fig. 5. Influence of the number of milliseconds of electroporation in *S. bayanus* viability.
(A) *S. bayanus* viability (percentage of cells excluding propidium iodide) after 0, 20, 25 or 30 ms of electroporation with 2500 V/cm, or 12 ms with 5000 V/cm. The values presented are for cells with Fluo-4 AM, but without addition of ethanol or $CaCl_2$. (B) *S. bayanus* viability after 20 (●), 25 (■) or 30 ms (▲) of electroporation with 2500 V/cm, as a function of increasing ethanol shock concentrations (v/v). The values presented are for cells with Fluo-4 AM, without addition of $CaCl_2$. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown. The response wasn't significantly linear in all conditions ($p>0.05$ in linearity test), and the slopes weren't significantly different from zero ($p>0.05$), except in the 30 ms condition ($p<0.05$).
The linear regression equations are $y = -0.0327x + 88.918$; $y = 0.036x + 80.923$; $y = 0.0916x + 70.01$, for 20, 25 and 30 ms, respectively.

This is easily explained by the fact that electroporation creates transient hydrophilic pores in cell membranes. These pores allow Fluo-4 AM to penetrate yeast cells, but when cells are subjected to electroporation for longer times or higher voltages, some cells could not reseal totally those temporary pores, being irreversibly permeable to propidium iodide[60]. The lowest viability percentage was reported for the 5000 V/cm condition, which was immediately set aside (Fig. 5A).

It is also important to address if cell viability is impaired by increasing ethanol shock concentrations, in order to be sure that the variation in fluorescence values detected were not due to cells leaking more or less intracellular Ca^{2+}, as well as the cleaved Fluo-4 AM, to the extracellular buffer. In fact, cell viability was not significantly affected by ethanol shock concentration, in the conditions of 20 and 25 ms (slopes weren't significantly different from zero, with $p>0.05$) and 30 ms of electroporation (interpretation based on the graphical

representation, due to lack of significantly linear response), allowing confidence in the fluorescence results (Fig. 5B).

Other aspect that was important to address, was the percentage of cells that in fact were loaded with Fluo-4 AM and fluorescent. The flow cytometer results show that more time of electroporation, contributed to a higher percentage of total cells with Fluo-4 AM (both alive and dead/injured) (statistical significance wasn't calculated due to lack of significantly linear response), and that cells in the 5000 V/cm condition presented the highest percentage, which is consistent with the fluorescence results. Looking at the results concerning only dead/injured cells loaded with Fluo-4 AM, the same pattern appears, confirming that the condition of electroporation with 5000 V/cm wasn't the ideal (Fig. 6A and B).

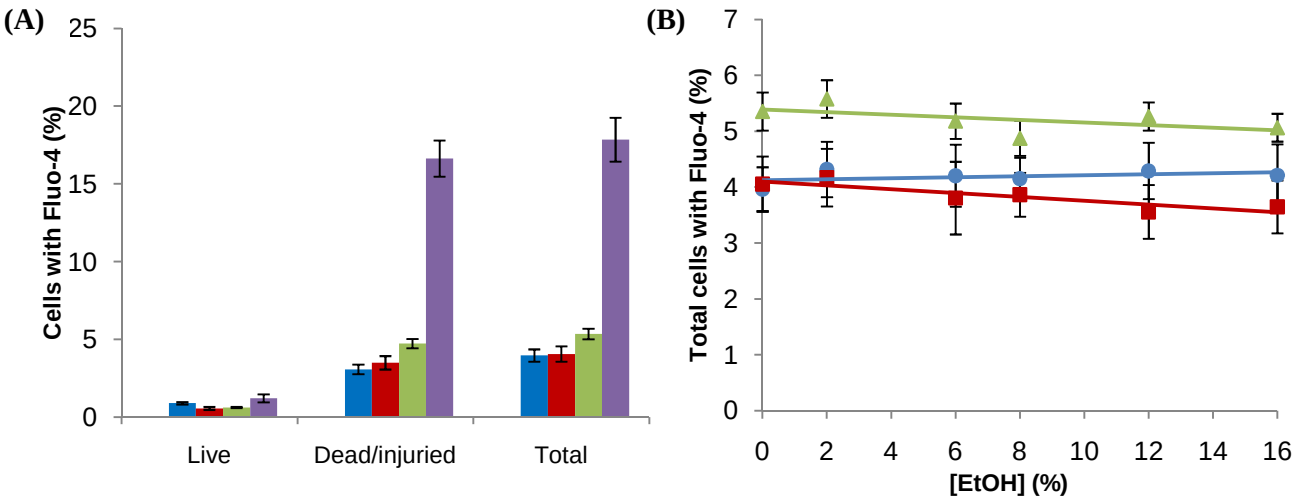


Fig. 6. Influence of the number of milliseconds of electroporation in the number of cells with Fluo-4 AM, in *S. bayanus.*
(A) Percentage of S. *bayanus* cells with Fluo-4 AM (live, dead/injured and total) after 0 (■), 20 (■), 25 (■) or 30 ms (■) of electroporation with 2500 V/cm, or 12 ms with 5000 V/cm (■). The values presented are for cells with Fluo-4 AM, but without addition of ethanol or $CaCl_2$. **(B)** Percentage of *S. bayanus* total cells with Fluo-4 AM after 20 (●), 25 (■) or 30 ms (▲) of electroporation with 2500 V/cm, as a function of increasing ethanol shock concentrations (v/v). The values presented are for cells with Fluo-4 AM, without addition of $CaCl_2$. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown. The response wasn't significantly linear in all conditions ($p>0.05$ in linearity test), and the slopes weren't significantly different from zero ($p>0.05$), except in the 25 ms condition ($p<0.05$).
The linear regression equations are y = 0.0087x + 4.1249; y = -0.0345x + 4.102; y = -0.0232x + 5.3887, for 20, 25 and 30 ms, respectively.

The results also show that the percentage of total (Fig. 6B), as well as live and dead/injured cells, with Fluo-4 AM wasn't significantly affected by increasing ethanol shock concentrations (for 20 and 30 ms the slopes weren't significantly different from zero, with $p>0.05$, and for 25 ms the interpretation was based on the graphical representation, due to lack of significantly linear response). Only around 1% of the cells were alive and loaded with Fluo-4 AM, which corresponds to $2,4 \times 10^5$ fluorescent live cells in each well of the 96-well microplate, which seems sufficient to detect fluorescence differences.

Since the number of cells effectively loaded with Fluo-4 AM was different between the electroporation conditions, to evaluate correctly which one confers the highest fluorescence,

it was necessary to normalize the fluorescence intensity values by the total number of cells with Fluo-4 AM in each individual condition, which gives the fluorescence intensity per cell with Fluo-4 AM. Both dead/injured and live cells with Fluo-4 AM were considered, because injured cells have compromised membranes, but may be able to recover and reproduce[53], as well as to respond to external stress with a Ca^{2+} response. With the dye used wasn't possible to distinguish between dead and injured cells, because propidium iodide enters cells whenever their membranes are damaged, being necessary a second dye, like SYTO 9, which is capable to label all yeast cells[53]. When both dyes are present in the same cell, propidium iodide causes a reduction of SYTO 9 stain fluorescence by fluorescence resonance energy transfer[61]. Since Syto9 wasn't available, and since its fluorescence is in the green wavelength, like Fluo-4 AM, it was chosen to use Fluo-4 AM and propidium iodide, abdicating from the possibility to distinguish between dead and injured cells.

The normalized results are shown in terms of fluorescence variation per cell with Fluo-4 AM (Fig. 7; Appendix 2 for fluorescence intensity values), and indicate that 25 ms of electroporation with 2500 V/cm was the best condition, having the highest increase in fluorescence variation per cell with Fluo-4 AM, as a function of ethanol shock concentration (slope significantly different from the 20 ms condition, with $p<0.05$). It's important to refer that despite the differences between 25 ms and 30 ms electroporation conditions weren't statistically significant ($p>0.05$), together with the flow cytometer results that points to higher cell viability in the 25 ms condition, this was chosen as the condition to be used in the rest of the experiments for this species. It was assumed that this condition was the best also for *S. bayanus* grown with 3% and 9% ethanol in growth medium. These results also reinforce the hypothesis that *S. bayanus* responds to ethanol shock with an increase of cytosolic Ca^{2+} concentration (slopes significantly different from zero, with $p<0.01$).

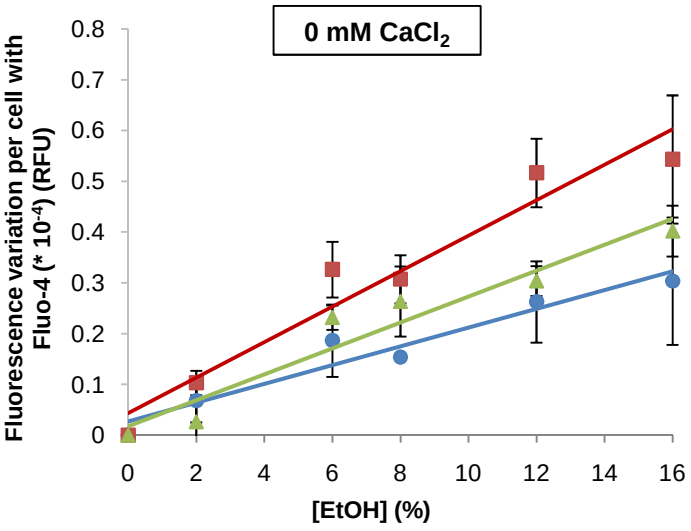


Fig. 7. Influence of the number of milliseconds of electroporation in *S. bayanus* Ca^{2+} response to ethanol shock, per cell with Fluo-4 AM.
Fluorescence variation per cell with Fluo-4 AM, after 20 (●), 25 (■) or 30 ms (▲) of electroporation with 2500 V/cm. The results are plotted as a function of ethanol shock concentration (v/v), for cells without addition of $CaCl_2$. The experiments were performed in triplicate, and the ±standard errors of the mean (SEM) are shown. Linear response was confirmed for all conditions ($p<0.01$ in linearity test and $p>0.05$ in deviation from linearity test), and all slopes were significantly different from zero ($p<0.01$).
The linear regression equations are $y = 0.0185x + 0.0272$; $y = 0.035x + 0.0432$; $y = 0.0255x + 0.0175$, for 20, 25 and 30 ms, respectively.

3.3.2. *S. cerevisiae* neotype strain (PYCC 4455)

S. cerevisiae neotype strain (PYCC 4455) required an additional optimization procedure, because when it was used the same Fluo-4 AM dilution factor (1:2), the fluorescence intensity was always under the autofluorescence values. This also happened when Fluo-4 AM was used non-diluted. So, the first step was the optimization of Fluo-4 AM concentration used in electroporation. The results show that the Fluo-4 AM dilution factor that results in the highest positive value of fluorescence intensity was 1:8 for cells grown with 0% ethanol, and was 1:32 for cells grown with 3% ethanol (Fig. 8).

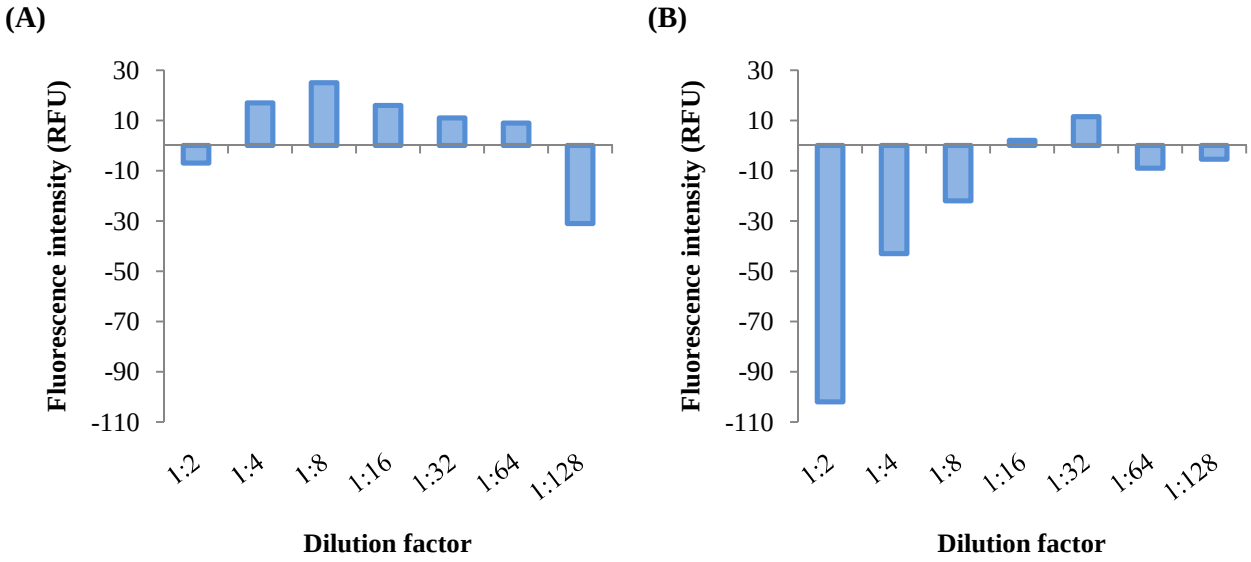


Fig. 8. Optimization of Fluo-4 AM concentration in *S. cerevisiae* neotype strain cells
Fluorescence intensity of *S. cerevisiae* cells (after autofluorescence subtraction) subjected to 10 ms of electroporation (2500 V/cm) with increasing Fluo-4 AM dilutions, after growth with (A) 0% or (B) 3% ethanol (v/v) in growth medium. The results are for cells without addition of ethanol or $CaCl_2$. The experiments were performed once.

One possible explanation for the necessity of using higher Fluo-4 AM dilutions in *S. cerevisiae* cells, when compared with *S. bayanus*, is possibly related with some characteristics of the Ca^{2+} sensitive dye. Fluo-4 AM is almost nonfluorescent, and these AM-ester groups are only cleaved intracellularly by endogenous esterases, releasing the Ca^{2+}-sensitive form[4]. After that, Fluo-4 AM is capable of suffer an increase of fluorescence intensity induced by free-Ca^{2+} binding[3]. Here, it seems that with the 1:2 dilution there was too much Fluo-4 AM that wasn't being cleaved, and therefore remaining non-fluorescent, probably masking or quenching the fluorescence of the fluorescent form and resulting in lower fluorescence intensities. This phenomenon is even more severe when cells were growing with 3% ethanol.

The mechanisms that underlie the less efficient cleavage in this species remain unexplained, but one can hypothesize that *S. cerevisiae* esterases are in lower number, have less affinity to those AM-ester groups, are less efficient, aren't working in the optimal

conditions, or can even being inhibited by high substrate concentrations. In fact, one study showed that *S. bayanus* and *S. cerevisiae* have different intracellular esterases[62], which could account for the differences in cleavage efficiency between those species. When the Fluo-4 AM concentration was lower, there was less non-fluorescent Fluo-4 AM, and was possible to detect the fluorescence. When the dilution factor increases even more, the Fluo-4 AM concentration inside yeast's cells was so low, that fluorescence intensity starts decreasing again.

The remaining optimization, to obtain the ideal number of milliseconds of electroporation, was done only for cells grown without ethanol, using the optimal Fluo-4 AM concentration. The cell fluorescence intensity was measured in a fluorescence spectrophotometer, after 5, 10 or 15 ms of electroporation with 2500 V/cm, and the results of fluorescence variation points to the 10 ms condition as the one with the highest fluorescence variation (interpretation was based on the graphical representation, because the linearity of the response wasn't tested, due to the experiments had been performed only once) (Fig. 9). The condition of 15 ms of electroporation was immediately discarded due to the low fluorescence intensity presented (Appendix 3 for fluorescence intensity values). Looking at the 10 ms condition, fluorescence values increase with increasing ethanol shock concentrations (slopes significantly different from zero, with $p<0.01$), but weren´t affected by the presence of $CaCl_2$ (interpretation was based on the graphical representation).

The flow cytometer results for cells without addition of 10 mM $CaCl_2$ show that a higher number of milliseconds of electroporation leaded to an increasing loss of viability, as expected (interpretation was based on the graphical representation, because the linearity of the response wasn't tested, due to the experiments had been performed only once) (Fig. 10). Cell viability was not significantly affected by ethanol shock concentration in the 5 ms condition (slope wasn't significantly different from zero, with $p>0.05$), but in the 10 and 15 ms conditions, the viability decreases slightly when 16% ethanol was added (interpretation was based on the graphical representation) (Fig. 10).

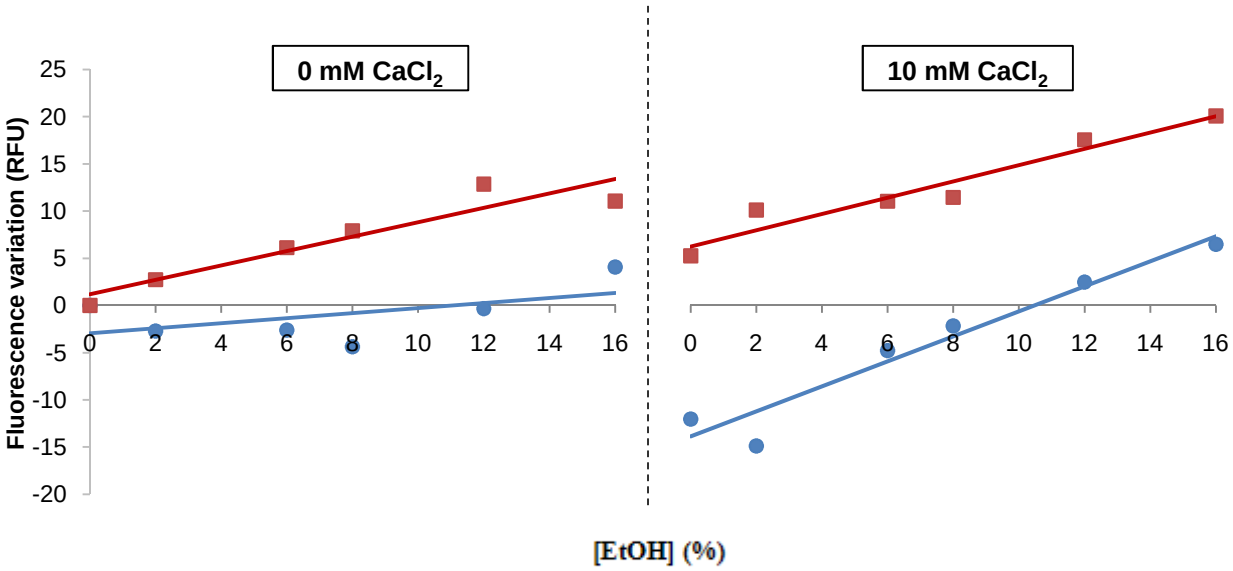


Fig. 9. Influence of the number of milliseconds of electroporation in *S. cerevisiae* neotype strain Ca^{2+} response to ethanol shock.
S. cerevisiae values of fluorescence variation, after 5 (●) or 10 ms (■) of electroporation with 2500 V/cm. The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations (v/v), with or without addition of 10 mM $CaCl_2$. The linearity of the response wasn´t tested, because the experiments were performed once. All slopes were significantly different from zero ($p<0.01$), except in the 5 ms condition, without $CaCl_2$ ($p>0.05$).
For 0 mM $CaCl_2$, the linear regression equations and R^2 values are y = 0.2684x - 2.9572 (R^2 = 0.2969); y = 0.7633x + 1.1832 (R^2 = 0.8853), for 5 and 10 ms condition, respectively. For 10 mM $CaCl_2$, the linear regression equations and R^2 values are y = 1.3251x - 13.864 (R^2 = 0.9402); y = 0.8622x + 6.2623 (R^2 = 0.9339), for 5 and 10 ms condition, respectively.

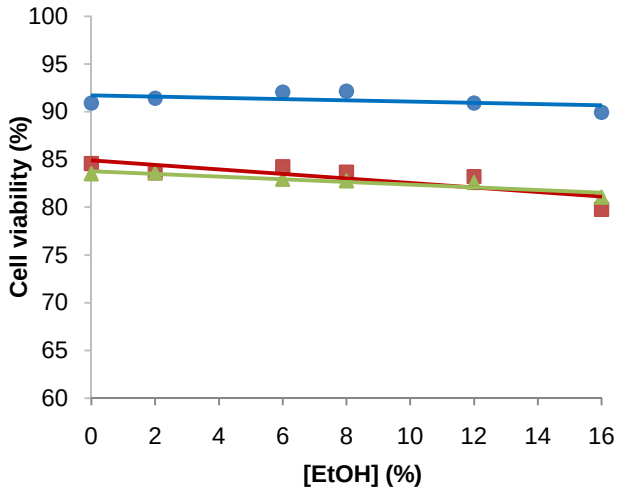


Fig. 10. Influence of the number of milliseconds of electroporation in *S. cerevisiae* neotype strain viability.
S. cerevisiae viability (percentage of cells excluding propidium iodide) after 5 (●), 10 (■) or 15 ms (▲) of electroporation with 2500 V/cm, as a function of increasing ethanol shock concentrations (v/v). The values presented are for cells with Fluo-4 AM, without addition of $CaCl_2$. The linearity of the response wasn´t tested, because the experiments were performed once. All slopes were significantly different from zero ($p<0.05$), except in the 5 ms condition, ($p>0.05$).
The linear regression equations and R^2 values are y = -0.0653x + 91.71 (R^2 = 0.2224); y = -0.2368x + 84.917 (R^2 = 0.6729); y = -0.1419x + 83.786 (R^2 = 0.8662), for 5, 10 or 15 ms, respectively.

Regarding the percentage of cells that in fact were loaded with Fluo-4 AM, the flow cytometer results show that 10 ms of electroporation leaded to the highest percentage of live, dead/injured and total cells with Fluo-4 AM, and the 15 ms condition as being the less efficient in cell loading (Fig. 11A). The results also show that the percentage of total (Fig. 11B), as well as live and dead/injured cells with Fluo-4 AM, decreased with the addition of ethanol, but was quite independent of its concentration, for all electroporation conditions, allowing to have confidence in the fluorescence results (interpretation was based on the graphical representation, due to the non-linear nature of the results). This result is in

conformity with the fact that *S. cerevisiae* is less resistant to ethanol stress, unlike *S. bayanus*, which cell viability was not significantly affected by ethanol concentration.

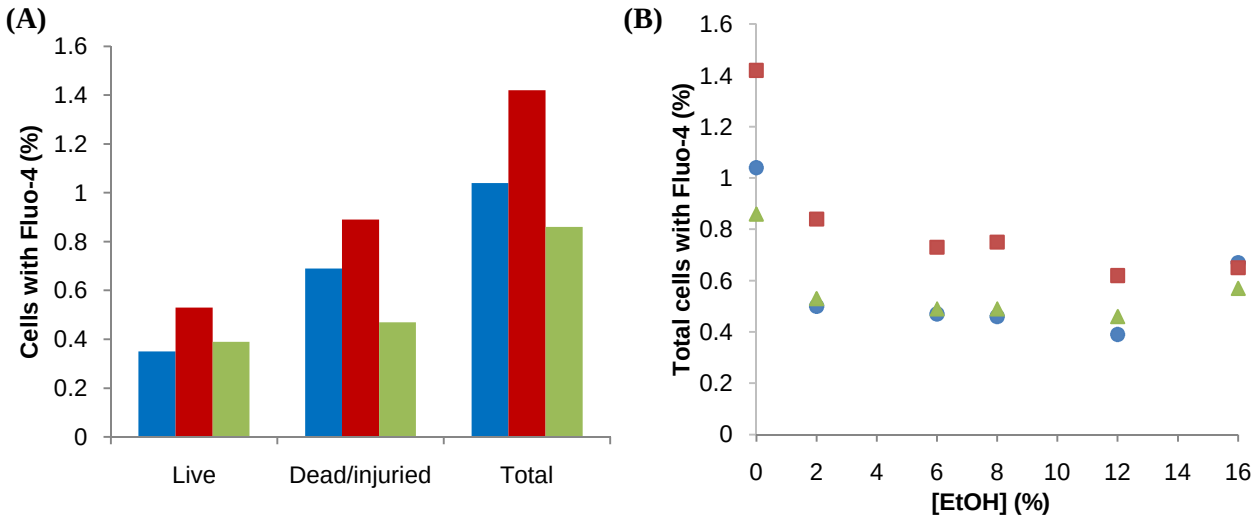


Fig. 11. Influence of the number of milliseconds of electroporation in the number of cells with Fluo-4 AM, in *S. cerevisiae* neotype strain.
(A) Percentage of S. *cerevisiae* cells with Fluo-4 AM (live, dead/injured and total) after 5 (■), 10 (■) or 15 ms (■) of electroporation with 2500 V/cm. The values presented are for cells with Fluo-4 AM, but without addition of ethanol or $CaCl_2$. (B) Percentage of *S. cerevisiae* total cells with Fluo-4 AM after 5 (●), 10 (■) or 15 ms (▲) of electroporation with 2500 V/cm, as a function of increasing ethanol shock concentrations (v/v). The values presented are for cells with Fluo-4 AM, without addition of $CaCl_2$. The experiments were performed once. Here, a linear regression wasn't applied, due to the non-linear nature of the results.

The normalized results confirm that 10 ms of electroporation with 2500 V/cm was the best condition, having the highest increase in fluorescence variation per cell with Fluo-4 AM (was the only condition with a slope significantly different from zero, with $p<0.01$) (Fig. 12), despite the lower fluorescence intensity (Appendix 4), as a function of ethanol shock concentration. So, this condition was chosen as the one to be used in the rest of the experiments for this species. It was assumed that this condition is the ideal also for *S. cerevisiae* grown with 3% ethanol. Again, these results are consistent with the hypothesis that both species respond to ethanol shock with an increase of cytosolic Ca^{2+} concentration.

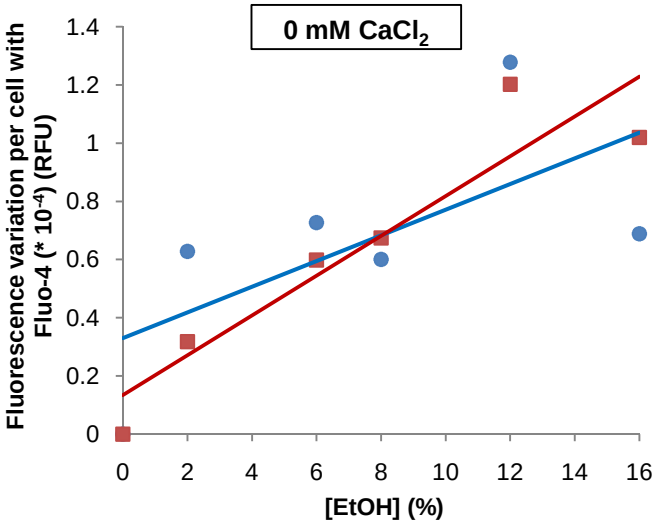


Fig. 12. Influence of the number of milliseconds of electroporation in *S. cerevisiae* neotype strain Ca^{2+} response to ethanol shock, per cell with Fluo-4 AM. Fluorescence variation per cell with Fluo-4 AM, after 5 (●) or 10 ms (■) of electroporation with 2500 V/cm. The results are plotted as a function of ethanol shock concentration (v/v), for cells without addition of $CaCl_2$. The linearity of the response wasn´t tested, because the experiments were performed once. The slope of the 10 ms condition was significantly different from zero ($p<0.01$), but the slope of the 5 ms condition wasn't ($p>0.05$). The linear regression equations and R^2 values are $y = 0.0442x + 0.3297$ ($R^2 = 0.4276$); $y = 0.0684x + 0.1336$ ($R^2 = 0.8691$), for 5 and 10 ms, respectively.

3.4. Control assays with cutinase enzyme

In order to exclude the hypothesis that ethanol could be interacting unspecifically with Fluo-4 AM, creating an artefact in the fluorescence results, a control assay was designed. The results show that cutinase effectively cleaved Fluo-4 AM, because it becomes capable of respond to the presence of Ca^{2+} with an increase of the fluorescence (Fig 13).

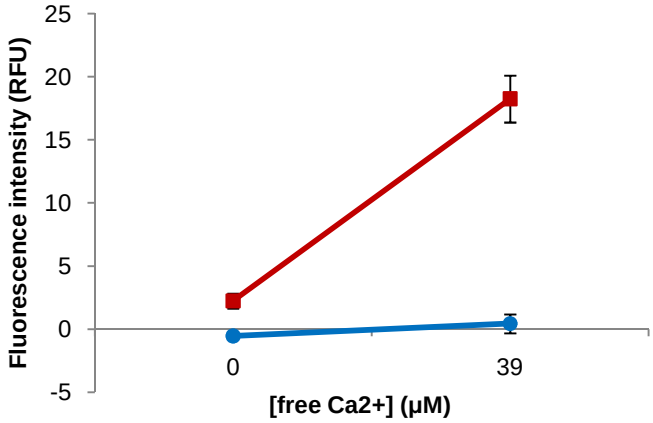


Fig. 13. Influence of esterase cleavage in Fluo-4 AM fluorescence.
Fluorescence intensity values for Fluo-4 AM incubated with cutinase (■) or with only phosphate buffer (●), as a function of free Ca^{2+} concentration. The autofluorescence values were subtracted to all results. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown.

Using Fluo-4 AM cleaved with cutinase, it's visible that ethanol didn't had an effect in fluorescence, in the absence of cells, when comparing with water, even when Ca^{2+} was added. Only the condition with 16% ethanol and 39 µM of free Ca^{2+} presented a slightly increase in fluorescence, when comparing with water (Fig. 14). So, in experiments with yeast cells, the conclusions for this last condition have to be made with some caution but, in general, the increase in fluorescence after ethanol addition was due to a true response from the cell, and not to an unspecific interaction between Fluo-4 AM and ethanol.

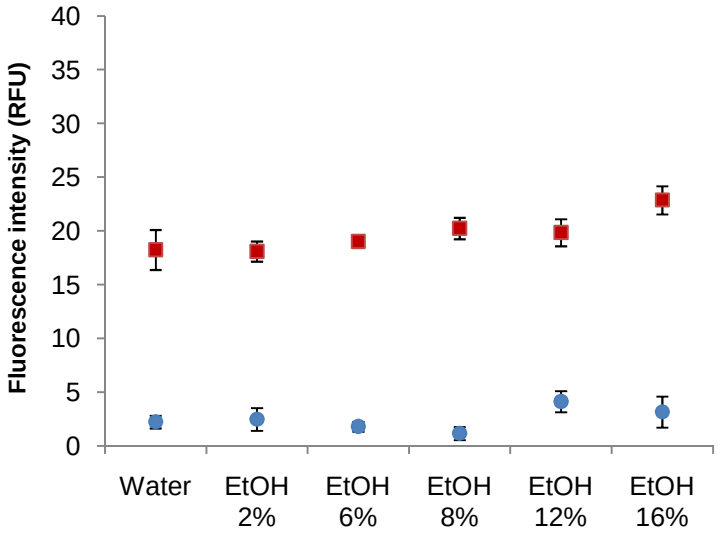


Fig. 14. Influence of ethanol in Fluo-4 AM fluorescence.
Fluorescence intensity values for Fluo-4 AM incubated with cutinase, with addition of 0 µM (●) or 39 µM free Ca^{2+} (■), as a function of ethanol concentration. The autofluorescence values were subtracted to all results. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown.

3.5. Effect of growth with ethanol in yeast's Ca^{2+} response

Using the optimal conditions defined earlier for both species, *S. bayanus* cells grown in the presence of 0, 3 or 9% ethanol (v/v), and *S. cerevisiae* neotype strain (PYCC 4455) grown in the presence of 0 or 3% ethanol (v/v) were analyzed. Their fluorescence after electroporation with Fluo-4 AM and subsequent addition of different ethanol and Ca^{2+} concentrations was measured. The main goals were to verify if there was an increase in cytosolic Ca^{2+} concentration after an ethanol shock, and if so, what was the provenience of that Ca^{2+}. Also, it was interesting to analyze if growing in the presence of ethanol, which presumably activates the stress response mechanisms, had some effect in the variation of cytosolic Ca^{2+} concentration in response to a posterior ethanol shock. It's already known that when yeast are pre-exposed to a sublethal amount of a stressing agent like ethanol, it can activate stress response mechanisms that result in a transient resistance to higher levels of ethanol[1], which can lead to a different pattern of Ca^{2+} response.

The fluorescence variation results show that both species responded to the shock with increasing ethanol concentrations, with increasing fluorescence intensity and also fluorescence variation (all slopes were significantly different from zero, with $p<0.01$) (Fig. 15; Appendix 5 for fluorescence intensity values), and therefore with an increase of cytosolic Ca^{2+} concentration. Analyzing these results, it appears that the response could occur in the absence of external Ca^{2+}, and wasn't significantly improved when external Ca^{2+} was added, because for all electroporation conditions, the slopes weren't significantly different when $CaCl_2$ was added ($p>0.05$). Comparing both species (Fig. 15), *S. bayanus* presented the highest fluorescence intensity and variation, with cells grown with 9% ethanol having the highest fluorescence variation of all growth conditions (slope significantly different from the 0 and 3% ethanol conditions without $CaCl_2$ addition, with $p<0.01$; when 10 mM $CaCl_2$ was added, the elevation was significantly different from the 0% ethanol condition, with $p<0.05$). *S. cerevisiae* cells grown with 3% ethanol have shown a slightly higher fluorescence variation than cells grown without ethanol, for the 0 mM Ca^{2+} condition (slopes weren't significantly different, but the elevations were, with $p<0.01$) (Fig. 15). This could indicate that cells grown with 9% ethanol (*S. bayanus*) and 3% ethanol (*S. cerevisiae*) responded more to ethanol shock, than cells grown without ethanol. One cannot exclude the hypothesis that *S. cerevisiae* lowest fluorescent intensity and variation, when compared with *S. bayanus* (slopes were significantly different, when comparing the same ethanol concentrations, with $p<0.05$), may also be caused by the fact that protocol optimization wasn't so successful for *S. cerevisiae*, as it had been for *S. bayanus*. So, it will be considered that results in terms of fluorescence variation per cell with Fluo-4 AM give a more real perspective of how the cells are responding to ethanol shock, than the raw fluorescence intensity values. In fact, these patterns will suffer alterations when fluorescence per cell results are analyzed ahead.

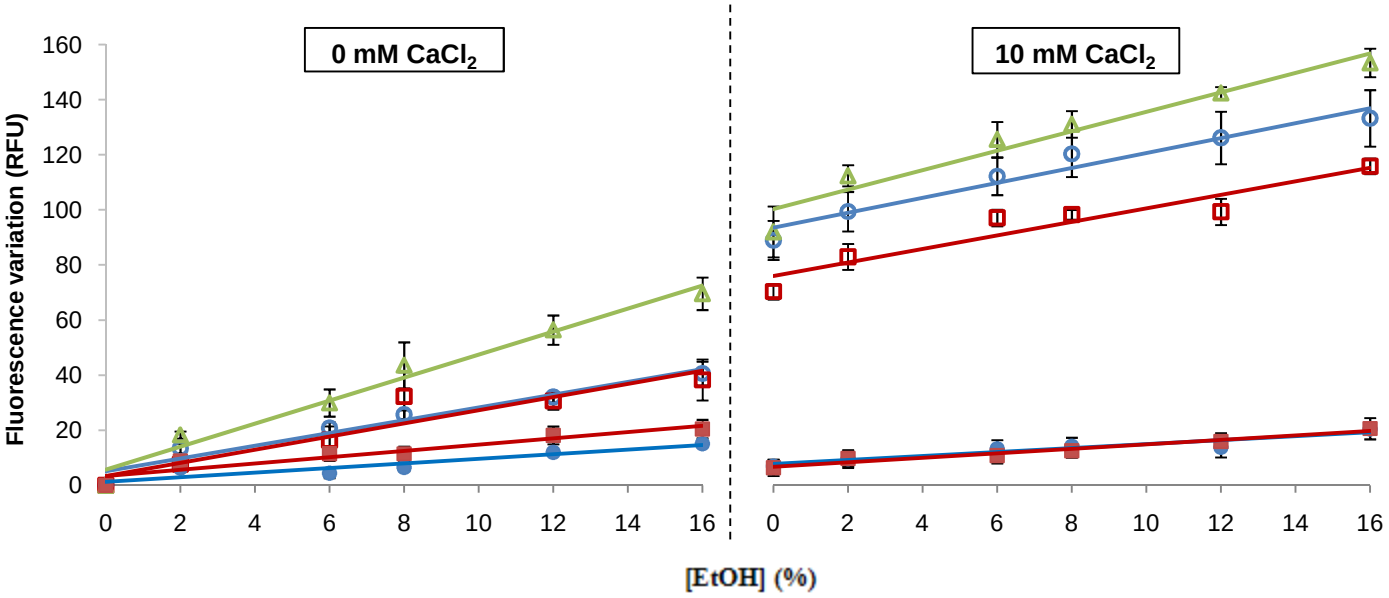


Fig. 15. Influence of growth with ethanol in *S. bayanus* and *S. cerevisiae* neotype strain Ca^{2+} responses to ethanol shock.
S. bayanus (open symbols) and *S. cerevisiae* (closed symbols) values of fluorescence variation, after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae*) of electroporation with 2500 V/cm, for cells grown in the presence of 0% (○, ●), 3% (□, ■) or 9% (△) ethanol (v/v). The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations (v/v), with or without addition of 10 mM $CaCl_2$. The results for *S. bayanus* cells growing in the absence of ethanol were from 6 replicates, while all other experiments were performed in triplicate. The ±standard errors of the mean (SEM) are shown. Linear response was confirmed for all conditions ($p<0.01$ in linearity test and $p>0.05$ in deviation from linearity test), except for S. cerevisiae grown with 0% ethanol and without addition of $CaCl_2$ ($p<0.05$ in deviation from linearity test). All slopes were significantly different from zero ($p<0.01$).
S. bayanus linear regression equations for 0 mM $CaCl_2$ are y = 2.3118x + 5.1339; y = 2.3896x + 3.3435; y = 4.1781x + 5.6616, for 0, 3 and 9% ethanol growth condition, respectively. *S. bayanus* linear regression equations for 10 mM $CaCl_2$ are y = 2.7117x + 93.466; y = 2.4528x + 75.971; y = 3.5251x + 100.26, for 0, 3 and 9% ethanol growth condition, respectively. *S. cerevisiae* linear regression equations for 0 mM $CaCl_2$ are y = 0.8382x + 1.2246; y = 1.1429x + 3.3556, for 0 and 3% ethanol growth condition, respectively. *S. cerevisiae* linear regression equations for 10 mM $CaCl_2$ are y = 0.7199x + 7.7378; y = 0.816x + 6.6891, for 0 and 3% ethanol growth condition, respectively.

The results from flow cytometry (Fig. 16 A and B) show that the highest viability values for *S. bayanus* (only analyzed for 0 mM $CaCl_2$ condition) were for the condition of growth with 3% ethanol, but for *S. cerevisiae* it happened in the 0% ethanol growth condition (interpretation based on the graphical representation, due to lack of significantly linear response). The *S. bayanus* result of higher viability in 3% ethanol growth condition was quite unexpected, because it has already been pointed that ethanol impairs growth and viability. Maybe the membrane's alterations induced by the growth in the presence of ethanol[23], also contributed to a higher resistance to damage caused by electroporation. Besides that, shock with different ethanol concentrations doesn't seem to affect cell viability, in each species or growth condition, except for *S. cerevisiae* grown without ethanol, that was less resistant to higher ethanol concentrations (12% and 16%), but just in the condition with $CaCl_2$ (interpretation based on the graphical representation, due to lack of significantly linear response). The aspect of *S. cerevisiae* being less resistant to high ethanol concentrations, when grown without ethanol in the growth medium, is easily explained by the fact that only

growing in the presence of ethanol results in a transient resistance to higher levels of ethanol[1]. But, one should expect that the addition of Ca^{2+} would at least result in the same viability values that were registered in the condition without Ca^{2+} suplementation, and never lower, because it was already described that Ca^{2+} supplementation during growth increased yeast's ethanol tolerance[26].

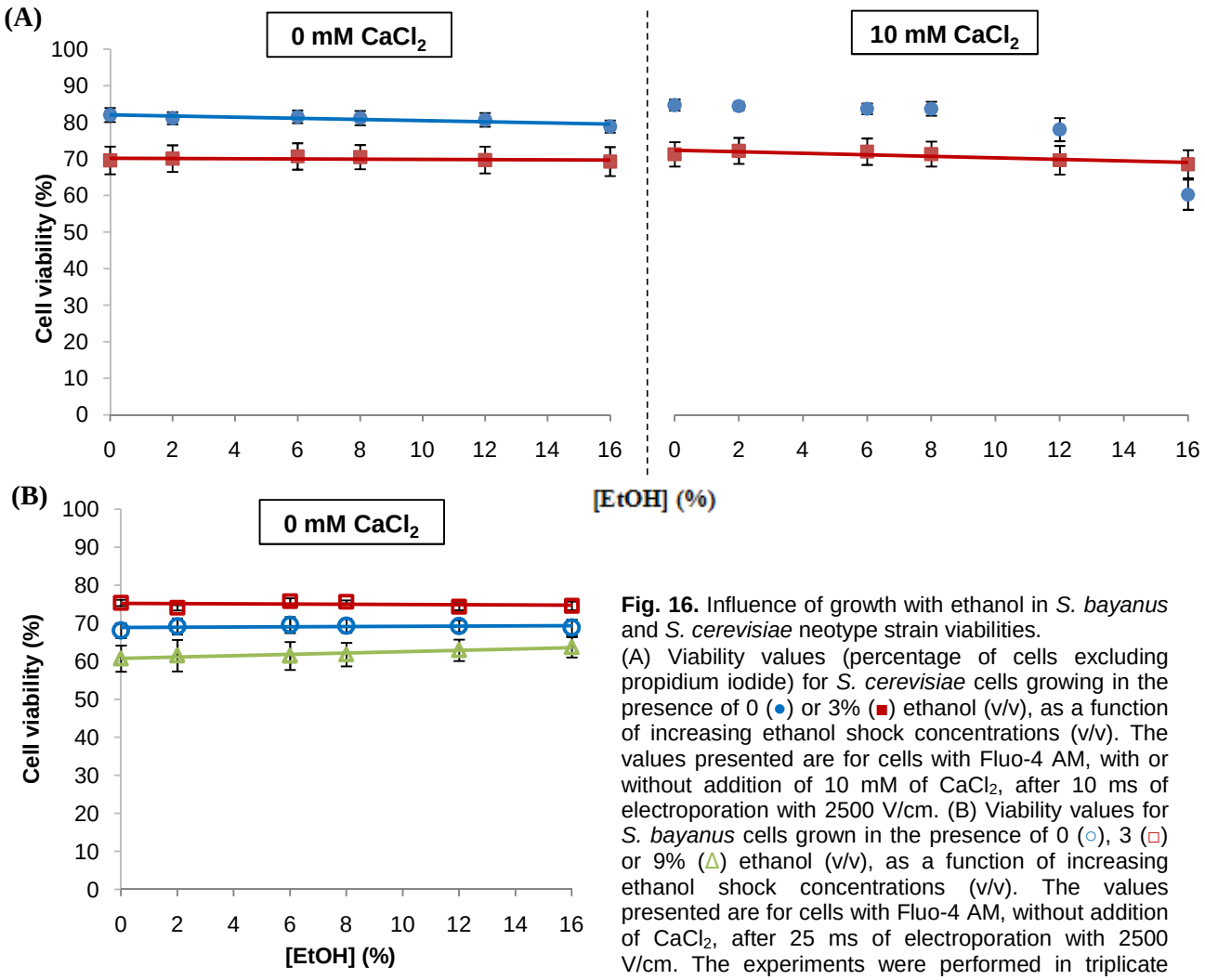


Fig. 16. Influence of growth with ethanol in *S. bayanus* and *S. cerevisiae* neotype strain viabilities. (A) Viability values (percentage of cells excluding propidium iodide) for *S. cerevisiae* cells growing in the presence of 0 (●) or 3% (■) ethanol (v/v), as a function of increasing ethanol shock concentrations (v/v). The values presented are for cells with Fluo-4 AM, with or without addition of 10 mM of $CaCl_2$, after 10 ms of electroporation with 2500 V/cm. (B) Viability values for *S. bayanus* cells grown in the presence of 0 (○), 3 (□) or 9% (Δ) ethanol (v/v), as a function of increasing ethanol shock concentrations (v/v). The values presented are for cells with Fluo-4 AM, without addition of $CaCl_2$, after 25 ms of electroporation with 2500 V/cm. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown. The response wasn't significantly linear in all conditions ($p>0.05$ in linearity test). Only the slopes for *S. bayanus* grown with 0% and 3% ethanol, and *S. cerevisiae* grown with 3% ethanol, without $CaCl_2$ addition, weren't significantly different from zero ($p>0.05$).
For *S.* cerevisiae, the linear regression equations for 0 mM of $CaCl_2$ are y = -0.1595x + 82.086; y = -0.0302x + 70.207, for 0 and 3% ethanol, respectively. The linear regression equations for 10 mM of $CaCl_2$ is y = -0.2048x + 72.343, for 3% ethanol. For *S.* bayanus, the linear regression equations are y = 0.029x + 68.852; y = -0.0277x + 75.191; y = 0.1755x + 60.729, for 0, 3 and 9% ethanol, respectively.

The analysis of the percentage of cells with Fluo-4 AM shows that *S. cerevisiae* cells had always fewer cells with Fluo-4 AM than *S. bayanus*, which again could be due to the problem in protocol optimization, or to intrinsic characteristics of yeast's cell membranes and intracellular esterases[62] (Fig. 17). For *S. bayanus* cells, the condition of growth with 9% ethanol had the highest percentage of total cells with Fluo-4 AM. On the other hand, cells grown with 3% ethanol presented the lowest percentages. These differences could explain

why cells grown with 9% ethanol had the highest fluorescence variation values. The highest number of cells loaded with Fluo-4 AM in the 9% ethanol condition could be explained also by the plasma membrane alterations induced by growth with ethanol, making it more permeable to Fluo-4 AM, than cells grown with 0% ethanol[23]. In the case of the *S. cerevisiae* cells, there was a higher percentage of cells with Fluo-4 AM in the condition of growth without ethanol, when compared with the 3% ethanol growth condition, which is compatible with the fluorescence intensity results (Appendix 5).

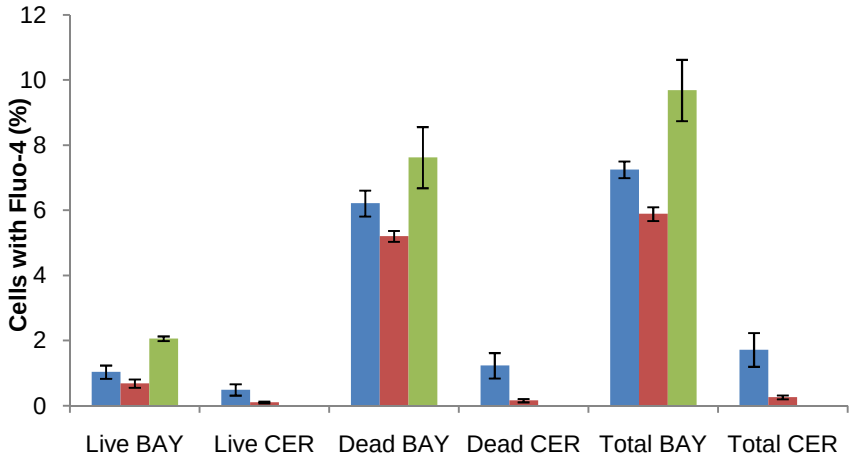


Fig. 17. Influence of growth with ethanol in the number of cells with Fluo-4 AM, in *S. bayanus* and *S. cerevisiae* neotype strain. Percentage of *S. bayanus* (BAY) and *S. cerevisiae* (CER) cells with Fluo-4 AM (live, dead/injured and total) after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae)* of electroporation with 2500 V/cm, for cells grown in the presence of 0 (■), 3 (■) or 9% (■) ethanol (v/v). The values presented are for cells with Fluo-4 AM, but without addition of ethanol or $CaCl_2$. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown.

In order to take correct conclusions from the fluorescence results, it's necessary to take into account the different percentage of fluorescent cells that each species and growth condition had. Since there aren't cytometry results for *S. bayanus* with addition of 10 mM $CaCl_2$, the response in the presence of Ca^{2+} will only be analyzed for *S. cerevisiae*.

The analysis of the results confirms that, for both species in all growth conditions, the fluorescence variation (Fig. 18 A; Appendix 6 for fluorescence intensity values) per cell with Fluo-4 AM was always higher with increasing ethanol shock concentrations (all slopes were significantly different from zero, with $p<0.05$). These results seem to indicate that *S. bayanus* and *S. cerevisiae* cells grown in the presence of 0, 3 or 9% ethanol were all capable of responding to ethanol shock with an increase of cytosolic Ca^{2+} concentration, as been hypothesized.

Focusing on *S. bayanus* results, there were no significant differences in slopes and elevations between *S. bayanus* cells grown with different ethanol concentrations ($p>0.05$) (Fig. 18 B). The values of fluorescence variation per cell with Fluo-4 AM was always much lower than *S. cerevisiae* values (slopes were significantly different, with $p<0.05$) (Fig. 18 A). This could be easily explained by the fact that *S. bayanus* strain used in this study was selected for winemaking, being naturally more resistant to ethanol, as seen in their growth curves (Fig. 2 A). Possibly, this species doesn't need to respond so intensely to ethanol shock in order to trigger the stress response mechanisms. On the other hand, the strain of *S.*

cerevisiae used in this study is much less resistant to ethanol[16], as noted in the growth curve experiment (Fig. 2 B), needing to trigger the stress response mechanisms when ethanol is present, using Ca^{2+} as the second messenger.

The results for *S. cerevisiae* show that fluorescence variation per cell with Fluo-4 AM was significantly higher for cells grown with 3% ethanol, when compared with 0% ethanol growth condition (slopes were significantly different, with $p<0.01$), which means that these cells were responding in a more intense way to the ethanol shock, than cells grown without ethanol (Fig. 18A). But, the 3% ethanol growth results have to be taken carefully, because the fluorescence intensity values (Appendix 6) for lower ethanol shock concentrations are under the autofluorescence values, probably because protocol optimization wasn't totally sucessfull. In future experiments, it should be important to improve the Fluo-4 AM delivery protocol in *S. cerevisiae*, in order to confirm if this pattern remains the same.

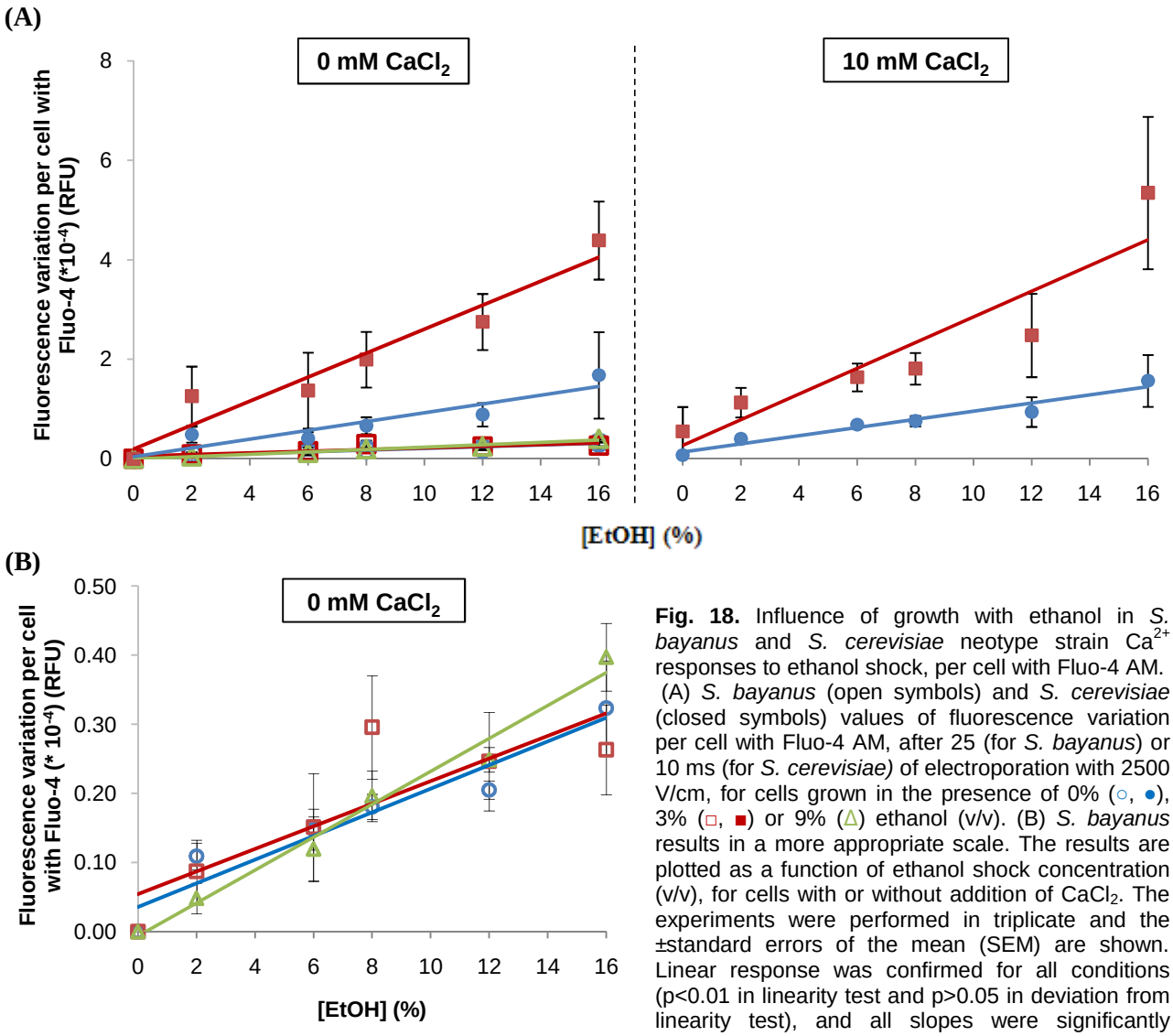


Fig. 18. Influence of growth with ethanol in *S. bayanus* and *S. cerevisiae* neotype strain Ca^{2+} responses to ethanol shock, per cell with Fluo-4 AM. (A) *S. bayanus* (open symbols) and *S. cerevisiae* (closed symbols) values of fluorescence variation per cell with Fluo-4 AM, after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae)* of electroporation with 2500 V/cm, for cells grown in the presence of 0% (○, ●), 3% (□, ■) or 9% (△) ethanol (v/v). (B) *S. bayanus* results in a more appropriate scale. The results are plotted as a function of ethanol shock concentration (v/v), for cells with or without addition of $CaCl_2$. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown. Linear response was confirmed for all conditions ($p<0.01$ in linearity test and $p>0.05$ in deviation from linearity test), and all slopes were significantly different from zero ($p<0.05$). *S. bayanus* linear regression equations for 0 mM $CaCl_2$ values are y = 0.0171x + 0.0356; y = 0.0163x + 0.0542; y = 0.0238x - 0.0063, for 0, 3 and 9% ethanol growth conditions, respectively. *S. cerevisiae* linear regression equations for 0 mM $CaCl_2$ are y = 0.0883x + 0.0404; y = 0.2408x + 0.1962, for 0 and 3% ethanol growth conditions, respectively. *S. cerevisiae* linear regression equations for 10 mM $CaCl_2$ are y = 0.0823x + 0.1344; y = 0.2586x + 0.2643, for 0 and 3% ethanol growth conditions, respectively.

It's important to refer that the variation in the fluorescence per cell was quite similar in the conditions with and without addition of external Ca^{2+}, for both growth conditions (0 and 3% ethanol), because the slopes weren't significantly different ($p>0.05$). This means that the response to ethanol stress seemed dependent only from Ca^{2+} released from intracellular stores, and wasn't improved with the addition of external Ca^{2+}. For *S. bayanus* it wasn't determined the fluorescence variation values per cell with Fluo-4 AM for the condition where 10 mM $CaCl_2$ was added, so wasn't possible to affirm with confidence that the same will happen in this species. But, one of the next experiments, where different external Ca^{2+} concentrations were added to the cells, will help to test if the origin of Ca^{2+} in *S. bayanus* is also the intracellular stores.

During the completion of this study, a paper[63] was published that presents some aspects also addressed here. Namely, the authors found that when *S. cerevisiae* was exposed to ethanol, it induced a rise in the cytoplasmic concentration of Ca^{2+}, which is in agreement with the present results. But, they defend that the Ca^{2+} flux was coming primarily from the extracellular solution, via the Cch1p-Mid1p Ca^{2+} influx channel, which seems to contradict these study results. It's important to highlight that the authors didn't test a control condition without addition of external Ca^{2+}, which raises some cautions about their conclusion that yeasts can't respond in the absence of external Ca^{2+}. Other problem is the fact that they only tested two deletion strains, respecting the genes of both subunits of the plasma membrane Ca^{2+} channel (CCH1 and MID1)[63]. To claim without doubt that Ca^{2+} spike didn't originate from intracellular stores, they should have tested at least other strain, with a deletion in YVC1, which is the gene that codes for a Ca^{2+} channel in the vacuolar membrane. The issue of the Ca^{2+} origin will be further tested in this work with the BY deletion strains for Ca^{2+} transporters.

3.6. Determination of intracellular Ca^{2+} concentration

As explained above, Fluo-4 AM binds free Ca^{2+} inside the cells, occurring an increase in its fluorescence. But, it's important to know for sure how much Ca^{2+} is involved in the responses already described. In order to determine that, it was used a Calcium Calibration Buffer Kit (Invitrogen).

The results show that existed a linear relationship between Ca^{2+} concentration and fluorescence intensity values ($p<0.001$), that was described by a different equation for each one of the species studied (Fig. 19). The two slopes weren't significantly different ($p>0.05$), showing that the response is similar in both species, but the elevations were different ($p<0.001$), which means that was necessary to use different equations to describe the responses.

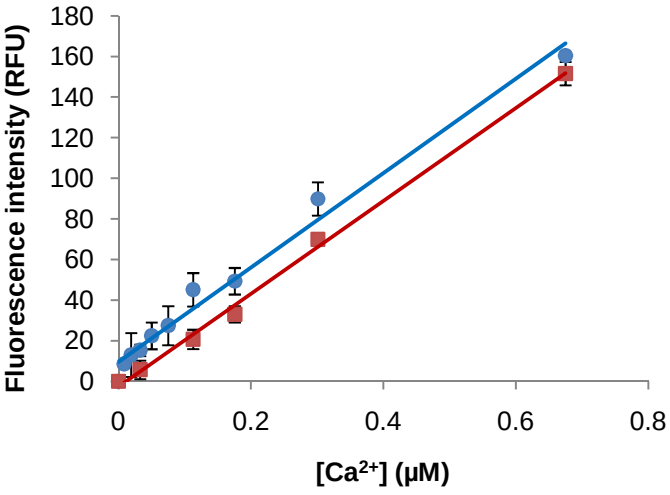


Fig. 19. Relation between Ca^{2+} concentration and fluorescence intensity values.
Relation between Ca^{2+} concentration and fluorescence intensity values, for *S. bayanus* (●) and *S. cerevisiae* neotype strain (PYCC 4455) (■). The autofluorescence values were subtracted to all results. The experiments were performed in duplicate for both species, and the ±standard errors of the mean (SEM) are shown. Linear response was confirmed for all conditions ($p<0.001$ in linearity test and $p>0.05$ in deviation from linearity test), and all slopes were significantly different from zero ($p<0.0001$).
S. bayanus and *S. cerevisiae* linear regression equations are y = 232.41x + 9,4928 and y = 228.48x -2.5397, respectively.

Knowing that, it was possible to calculate the real intracellular Ca^{2+} concentrations that were detected in the previous experiments, in terms of fluorescence intensity (Appendix 5), with *S. bayanus* and *S. cerevisiae* neotype strain grown in the presence of 0, 3 or 9% ethanol (Fig. 20).

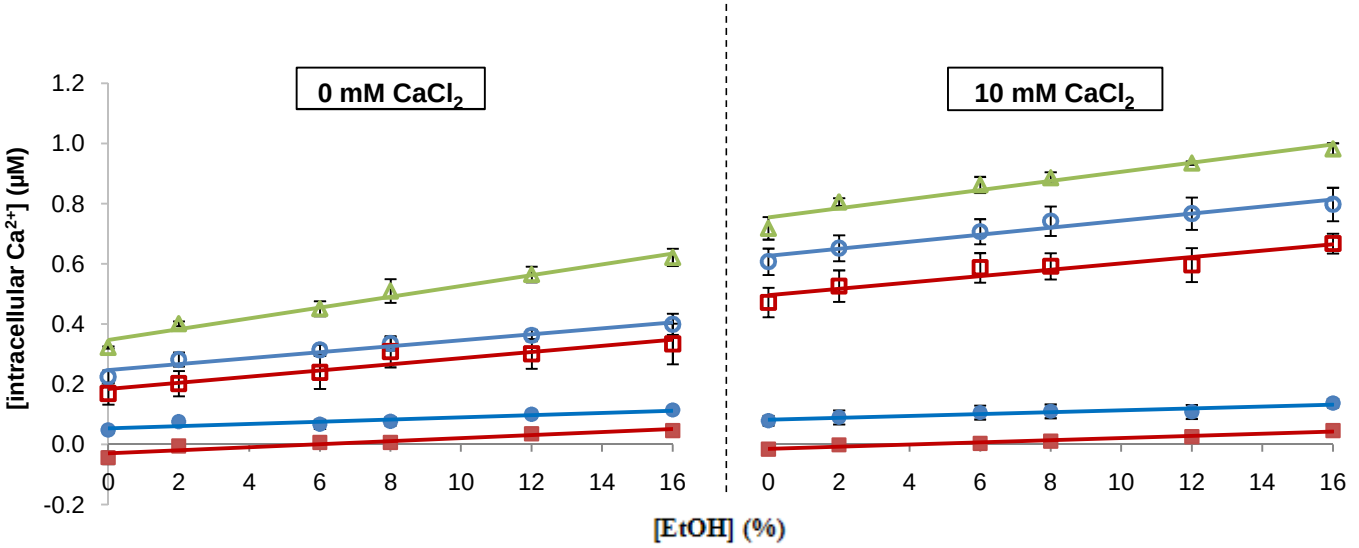


Fig. 20. Determination of intracellular Ca^{2+} concentration in *S. bayanus* and *S. cerevisiae* neotype strain.
S. bayanus (open symbols) and *S. cerevisiae* neotype strain (PYCC 4455) (closed symbols) values of intracellular Ca^{2+} concentration, for cells grown in the presence of 0% (○, ●), 3% (□, ■) or 9% (△) ethanol (v/v). The values were presented as a function of increasing ethanol shock concentrations (v/v), with or without addition of 10 mM $CaCl_2$. The results for *S. bayanus* cells growing in the absence of ethanol were from 6 replicates, while all other experiments were performed in triplicate. The ±standard errors of the mean (SEM) are shown. Linear response was confirmed for all conditions ($p<0.05$ in linearity test and $p>0.05$ in deviation from linearity test), except for *S. cerevisiae* grown with 0% ethanol and with 10 mM $CaCl_2$ addition ($p>0.05$ in linearity test). All slopes were significantly different from zero ($p<0.01$).
S. bayanus linear regression equations for 0 mM $CaCl_2$ are y = 0,0099x + 0,247; y = 0,0103x + 0,184; y = 0,018x + 0,3472, for 0, 3 and 9% ethanol growth condition, respectively. *S. bayanus* linear regression equations for 10 mM $CaCl_2$ are y = 0,0117x + 0,627; y = 0,0106x + 0,4965; y = 0,0152x + 0,7542, for 0, 3 and 9% ethanol growth condition, respectively. *S. cerevisiae* linear regression equations for 0 mM $CaCl_2$ are y = 0,0037x + 0,0538; y = 0,005x - 0,029, for 0 and 3% ethanol growth condition, respectively. *S. cerevisiae* linear regression equations for 10 mM $CaCl_2$ are y = 0,0032x + 0,0823; y = 0,0036x - 0,0144, for 0 and 3% ethanol growth condition, respectively.

The intracellular Ca^{2+} concentrations calculated were in the normal range for yeasts. Usually, they maintain cytosolic Ca^{2+} concentrations at very low levels, around 50-200 nM[52], but after a stimulus that promote a Ca^{2+} signal, the intracellular Ca^{2+} concentrations

increases 10 to 100-fold over the basal level. Considering an average basal level of 0.1 µM, Ca^{2+} concentrations can increase until 1-10 µM[31]. The results for *S. bayanus* show that cells in the basal conditions (0% ethanol shock) had intracellular Ca^{2+} concentrations values near the upper limit expected for unstimulated cells, except cells grown with 9% ethanol, that were slightly above. But, when stimulated by an ethanol shock, Ca^{2+} concentrations increased until the lower limit of the expected range. For *S. cerevisiae* neotype strain, the maximum Ca^{2+} concentration value obtained was for cells grown with 0% ethanol, and with addition of 10 mM $CaCl_2$ and 16% ethanol. But, even in this condition, the Ca^{2+} concentration value was in the range expected for a steady-state cell. Since the previous results show that cells were responding to ethanol stress, this low Ca^{2+} concentration level was due to the protocol optimization problems already refered, that resulted in a poor Fluo-4 AM cleavage in this species.

3.7. Effect of external Ca^{2+} concentration

The previous results indicate that the Ca^{2+} increase registered during the ethanol shock response is due to a release from intracellular stores. In order to test further this hypothesis, other experiment was performed, in which different external Ca^{2+} concentrations were added to the yeasts, which were all grown without ethanol in the growth medium. Besides that, it was also added 0% or 8% ethanol, or 8% ethanol with EDTA (EthyleneDiamineTetraacetic Acid) that is a chelating agent of metal ions. It's important to note that EDTA concentration in each condition was always equal to the Ca^{2+} concentration in the same condition, because the chelating reaction is complete when both components are in a 1:1 mole ratio. Therefore, in the conditions where EDTA was added, all the external Ca^{2+} was unavailable to be used by the cell, and also unable to bind Fluo-4 AM, because it wasn't in a free form[64].

Due to the non-linear nature of the results in both species, interpretations were only based on the graphical representations. The results for *S. bayanus* show that when 8% ethanol was added, the fluorescence intensity (Fig 21A) was always higher, for all external Ca^{2+} concentrations, when compared with 0% ethanol. This is in agreement with the hypothesis that ethanol shock promotes an increase of cytosolic Ca^{2+} concentration. But, the fluorescence variation results (Fig. 21B) show that the variation in fluorescence, with increasing external Ca^{2+} concentrations, wasn't significantly different between the conditions where 0 and 8% ethanol was added. This means that addition of external Ca^{2+} alone was capable of increasing intracellular Ca^{2+} concentrations[57]. So, external Ca^{2+} addition only created a new fluorescence intensity baseline for the cell, and when 8% ethanol was added, the fluorescence intensity increased even more, due to Ca^{2+} being released from intracellular stores, in a response to ethanol stress. This is a strong evidence that the Ca^{2+} necessary for *S. bayanus* to respond to an ethanol shock, comes from intracellular stores. A similar

response to an external stress has already been reported for yeasts responding to hypotonic shock. This stress generated a Ca^{2+} pulse, that was primarily generated from intracellular stores. Only after this first response, was that the sustained increase in cytosolic Ca^{2+} concentration depended upon Ca^{2+} entering from the extracellular medium[5].

Analyzing the condition with EDTA, regardless of external Ca^{2+} being needed or not to ethanol shock response, it was expected that fluorescence variation values were equal for different external Ca^{2+} concentrations, because EDTA concentrations were in a 1:1 mole ratio with Ca^{2+}, and therefore all added Ca^{2+} will become unavailable. In the case of external Ca^{2+} being needed to ethanol stress response, addition of EDTA should result in lower fluorescence intensity values (but similar between them), than in the situation where external Ca^{2+} wasn't essential. But, actually, the fluorescence variation values in the condition with EDTA decreased with increasing external Ca^{2+} concentrations. The only possible explanation was that EDTA was entering yeast cells and also chelating internal Ca^{2+}, that becomed unavaible to the ethanol stress response. This chelating reaction was stronger with increasing EDTA concentrations. One study showed that EDTA could in fact enter inside *S. cerevisiae* cells, forming chelation with Ca^{2+} and other divalent ions[65]. So, isn't possible to utilize the EDTA condition results to take further conclusions about the origin of Ca^{2+} that was being used in the ethanol shock response, because all Ca^{2+} (external and internal) was unavailable.

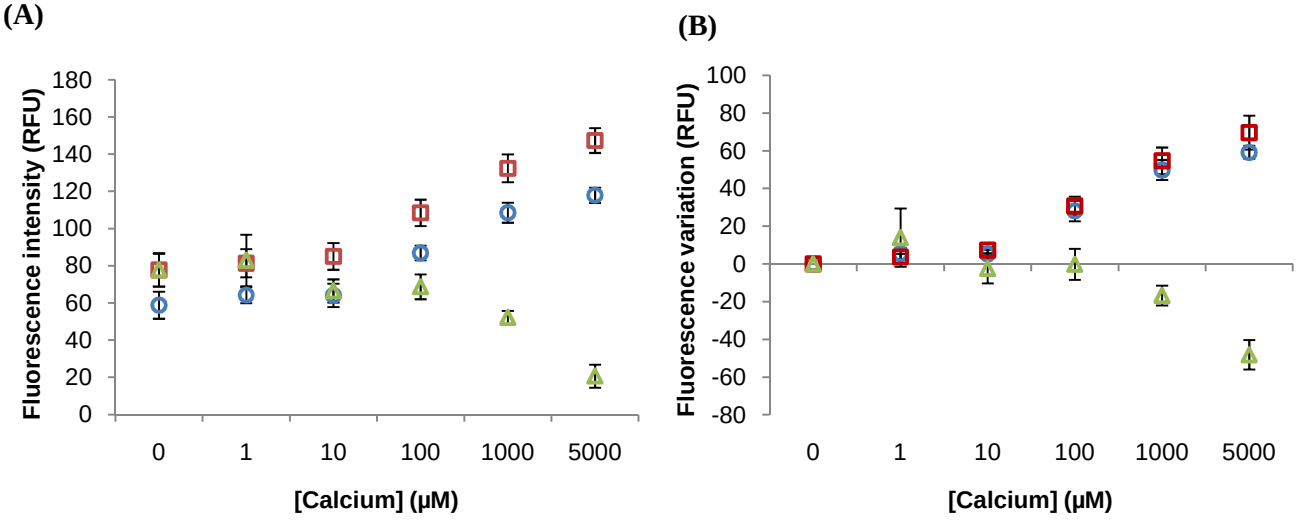


Fig. 21. Influence of external Ca^{2+} concentration in *S. bayanus* response.
(A) *S. bayanus* values of fluorescence intensity and (B) fluorescence variation, after 25 ms of electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v), and after addition of 0% (○) or 8% ethanol (□), or 8% ethanol + EDTA (△). The autofluorescence values were subtracted to all results, and then presented as a function of increasing Ca^{2+} concentrations added. The experiments were performed in triplicate and the ±standard errors of the mean (SEM) are shown. Here, a linear regression wasn't applied, due to the non-linear nature of the results.

In *S. cerevisiae* neotype strain (PYCC 4455) (Fig. 22 A and B), the results were more difficult to interpret, because the values weren't different between conditions, in most cases, due to high standard errors of the mean (SEM). The effect of EDTA in lowering the fluorescence intensity, when compared with the other conditions, was similar to what happened in *S. bayanus*, but less significant. And for most external Ca^{2+} concentrations, fluorescence variation wasn't different between the conditions without or with 8% ethanol, corroborating the hypothesis that external Ca^{2+} addition only created a new fluorescence intensity baseline. But, in the case *of S. cerevisiae*, more biological replicates were needed to take conclusions with higher confidence. Other sugestion for future experiments is to make measurements in the flow cytometer, in order to calculate fluorescence intensity values per cell with Fluo-4 AM, which could help to see more clear patterns in this species.

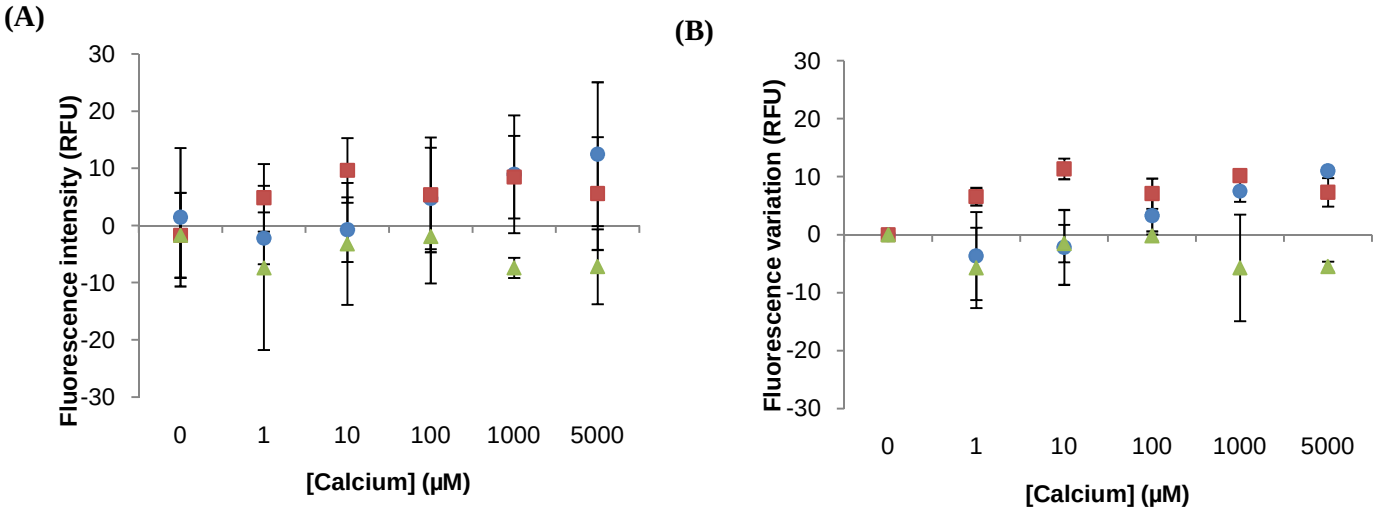


Fig. 22. Influence of external Ca^{2+} concentration in *S. cerevisiae* neotype strain response.
(A) *S. cerevisiae* neotype strain (PYCC 4455) values of fluorescence intensity and (B) fluorescence variation, after 10 ms of electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v), and after addition of 0% (●) or 8% ethanol (■), or 8% ethanol + EDTA (▲). The autofluorescence values were subtracted to all results, and then presented as a function of increasing Ca^{2+} concentrations added. The experiments were performed in duplicate and the ±standard errors of the mean (SEM) are shown. Here, a linear regression wasn't applied, due to the non-linear nature of the results.

3.8. Looking for evidences of GPCR activation

Other main objective of this study was to investigate if the increase in cytosolic Ca^{2+} concentration was due to the activation of a GPCR by ethanol. In *S. cerevisiae,* two distinct GPCR systems have been identified: one for glucose-sensing, and other for pheromone signaling[6]. Since ethanol could be a carbon source used by the yeasts[66], it's logical to first test if ethanol could be acting as an agonist of the glucose-sensing GPCR. It's also easier to test this GPCR, because one known agonist is glucose, and antagonist is mannose[6], which are more commonly available, than pheromones. But, because pheromone signaling GPCR act by calcineurin/Crz1p pathway, and ethanol can act by the same pathway[2], this GPCR is

also a good candidate for ethanol to act as an agonist. This aspect will only be tested later, using BY deletion strains for this GPCR gene. Of course that there is the possibility that ethanol isn't activating any of these GPCRs, acting through another pathway.

3.8.1. Glucose as agonist of glucose-sensing GPCR

First, it was tested if it's possible to detect the glucose-sensing GPCR activation and inactivation in terms of fluorescence intensity. With that in mind, an experiment was designed, where increasing glucose (agonist) and mannose (antagonist) concentrations were added to electroporated cells, previously grown without ethanol. Again, there was a condition without addition of external Ca^{2+}, and another where 10 mM Ca^{2+} were added.

Since most of the regressions weren't significantly linear, interpretations were only based on the graphical representations. For *S. bayanus* (Fig. 23A; Appendix 7 for fluorescence intensity values), the results show that only in the situation where 10 mM Ca^{2+} were added, there were differences between the presence or absence of antagonist. In this condition, fluorescence variation was always higher when mannose was absent (only glucose is present), than when both mannose concentrations were added. This shows that it's possible to see the antagonist effect of mannose, in terms of fluorescence. In terms of the effect of the agonist, it's harder to see. On one hand, when only glucose (without mannose) was present, fluorescence variation was higher. But on the other hand there wasn't the dose-response relationship that was expected, since fluorescence intensity (Appendix 7) should increase with increasing glucose concentrations[6], but in reality it remained almost stable. Probably, the reason for this to happen was the high external Ca^{2+} concentration that established a high fluorescence baseline, making the dose-response effect harder to see. One evidence in favor of that is the fact that in the 0 mM Ca^{2+} condition, was possible to see a slightly dose-response relationship. In future studies will be also important to test further glucose concentrations, lower than 100 mM, because a previous study showed that 20-30 mM glucose were necessary for half-maximal activation of Gpr1-dependent cAMP signalling[6].

Several studies showed that the increase in free intracellular Ca^{2+} concentration caused by glucose addition, was mainly due to an influx from external medium[43,56]. This explains why only in the 10 mM Ca^{2+} condition was possible to see that fluorescence variation induced by the agonist was higher that the one induced by the antagonist. The slightly dose-response relationship seen in the 0 mM Ca^{2+} condition could be due to some Ca^{2+} released from intracellular stores, or to residual Ca^{2+} existent in the solutions and material used.

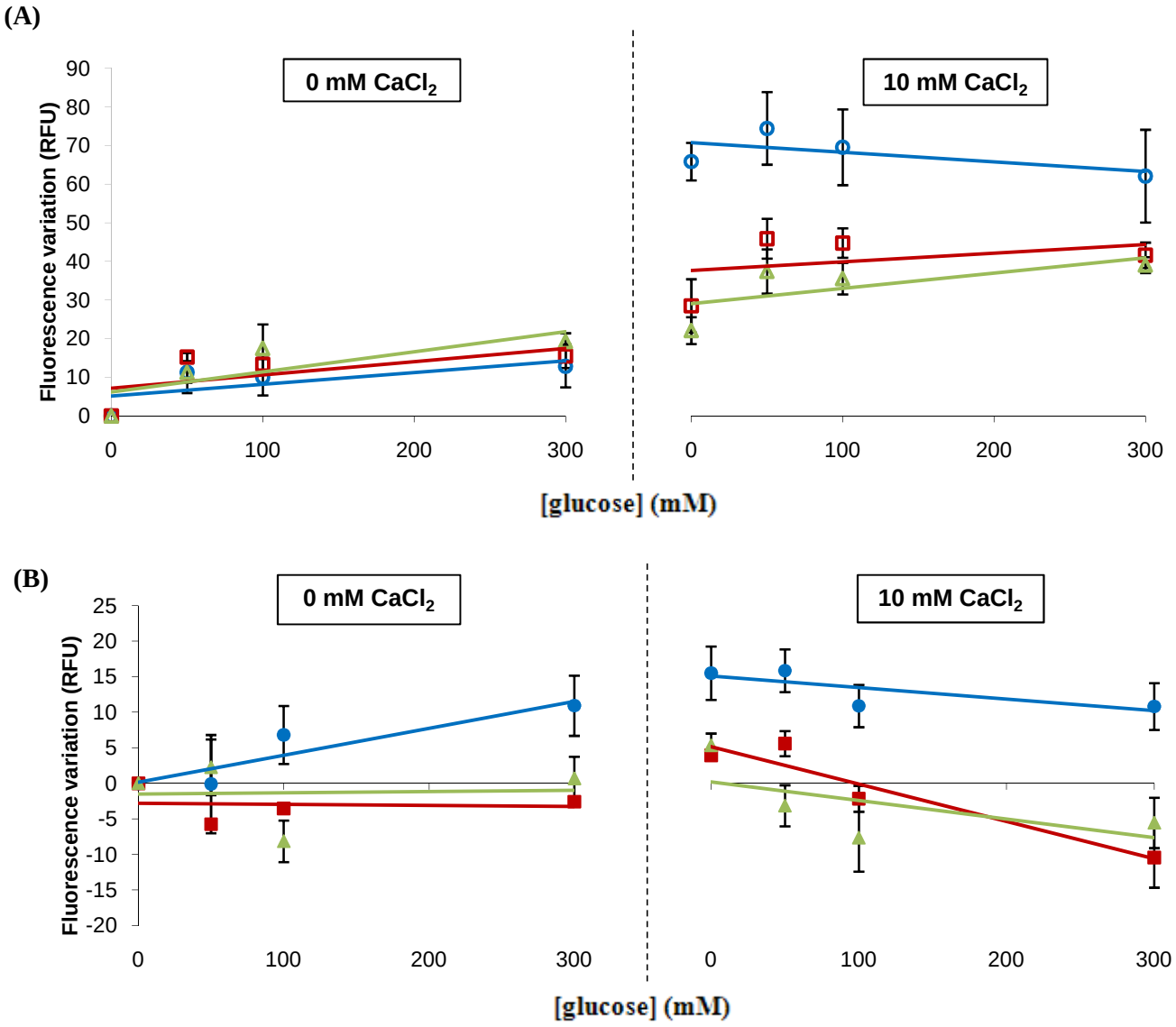


Fig. 23. Influence of glucose and mannose addition in *S. bayanus* and *S. cerevisiae* neotype strain Ca^{2+} response, in order to detect activation of the glucose-sensing GPCR.
(A) *S. bayanus* and (B) *S. cerevisiae* neotype strain (PYCC 4455) values of fluorescence variation, after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae)* of electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v), and after addition of 0 (○,●), 200 (□, ■), or 400 mM mannose (△, ▲). The autofluorescence values were subtracted to all results, and then presented as a function of increasing glucose concentrations, with or without addition of 10 mM $CaCl_2$. The experiments were performed in triplicate for *S. bayanus* and duplicate for *S. cerevisiae*, and the ±standard errors of the mean (SEM) are shown. The response wasn't significantly linear in all conditions ($p>0.05$ in linearity test), except for *S. bayanus* with addition of 400 mM mannose and 0 mM $CaCl_2$ ($p<0.05$) and *S. cerevisiae* with addition of 200 mM mannose and 10 mM $CaCl_2$ ($p<0.01$). Only the slope for *S. cerevisiae* with addition of 200 mM mannose and 10 mM $CaCl_2$ was significantly different from zero ($p<0.05$).
S. bayanus linear regression equations for 0 mM $CaCl_2$ are y = 0.0304x + 5.1056; y = 0.0345x + 7.153; y = 0.0519x + 6.1853, for 0, 200 and 400 mM mannose, respectively. *S. bayanus* linear regression equations for 10 mM $CaCl_2$ are y = -0.0249x + 70.766; y = 0.0225x + 37.639; y = 0.0397x + 29.032, for 0, 200 and 400 mM mannose, respectively. *S. cerevisiae* linear regression equations for 0 mM $CaCl_2$ are y = 0.0379x + 0.1413; y = -0.0016x - 2.7934; y = 0.0017x - 1.4927, for 0, 200 and 400 mM mannose, respectively. *S. cerevisiae* linear regression equations for 10 mM $CaCl_2$ are y = -0.0162x + 15.085; y = -0.0525x + 5.1372; y = -0.026x + 0.1792, for 0, 200 and 400 mM mannose, respectively.

The results for *S. cerevisiae* neotype strain (Fig. 23B; Appendix 7 for fluorescence intensity values) have a similar interpretation, except in the 0 mM Ca^{2+} condition, where fluorescence variation for the 0 mM mannose condition was higher than 200 and 400 mM mannose conditions. Considering the previous studies conclusions already refered[43,56], this

was quite unexpected. But, analysing carefully that previous studies fluorescence results, the glucose-induced Ca^{2+} signaling wasn't totally abolished in the absence of external Ca^{2+} [56], and they even suggest that cannot be excluded the release of Ca^{2+} from intracellular sources[43]. In future experiments, it could be interesting to analyze further if glucose-induced Ca^{2+} signaling is really dependent mainly from external Ca^{2+}.

In both species (Fig. 23 A and B), the 200 mM and 400 mM mannose concentrations didn't present differences, but it was expected that higher antagonist concentrations should result in lower fluorescence variations. In a previous study, were visible significant differences in the glucose induced cAMP signaling using these mannose concentrations[42], so we were expecting to also see differences in Ca^{2+} signalling.

It's also important to highlight that the possible types of antagonists are competitive, non-competitive and uncompetitive. In the competitive case, both agonist and antagonist bind to the same site on the receptor, so the action of the antagonist could be overcome using higher concentrations of the agonist[39]. In the non-competitive case, the binding sites for agonist and antagonist are different, so increasing doses of agonist will not overcome the antagonist effect[39]. An uncompetitive antagonist is when its action is contingent upon prior activation of the receptor by the agonist. In this case, the same concentration of antagonist blocks better higher concentrations of agonist, than lower concentrations[40].

A previous study has shown that mannose is, until now, the only antagonist known for this GPCR[42], but the antagonist type hasn't been yet identified. In the case of *S. bayanus*, fluorescence variation values when mannose was added were higher with increasing agonist concentrations, which points to mannose has being a competitive antagonist. But, in the case of *S. cerevisiae*, fluorescence variation when mannose is added decreases with increasing agonist concentration, in the condition with 10 mM Ca^{2+}, which is consistent with mannose being an uncompetitive antagonist. In order to clearly identify the antagonist type of mannose in both species, more experiments are needed, using more glucose and mannose concentrations.

3.8.2. Ethanol as a possible agonist of glucose-sensing GPCR

Since it was possible to see fluorescence variations with agonist and antagonist addition in both species, the next step was to see if ethanol could act as an agonist of the glucose-sensing GPCR.

If ethanol is an agonist of this GPCR, it will be possible to see the decrease in fluorescence caused by mannose. Since the buffer where yeasts were ressuspended had no glucose or sucrose, and cells were washed previously to remove all traces of growth medium, it's safe to claim that in this experiment there weren't any known agonists for this GPCR present in the cell suspension, besides ethanol, that was tested for its role as agonist.

The results with ethanol (Fig. 24; Appendix 8 for fluorescence intensity values) showed similar patterns for *S. bayanus* and *S. cerevisiae* neotype strain (PYCC 4455), when compared with the glucose results. More specifically, for *S. bayanus* (Fig. 24 A), there were significant differences between the presence or absence of antagonist only in the situation were 10 mM Ca^{2+} was added (elevations were significantly different, with $p<0.0001$), as already happened for glucose. In this condition, fluorescence variation was always lower when mannose was added, showing that ethanol could be acting as agonist of this GPCR. This time, it was possible to see the dose-response relationship in the 0 mM manose condition, because fluorescence variation increased with increasing ethanol shock concentrations (slope was significantly different from zero, with $p<0.05$). Again, there weren't significant differences between the two mannose concentrations (slopes and elevations weren't significanly different, with $p>0.05$).

The only unexpected result was the fact that in the 0 mM Ca^{2+} condition wasn't possible to see the antagonist effect (interpretation based on the graphical representation, due to lack of significantly linear response of 200 mM mannose condition). It was expected that, since ethanol stress response doesn't seem dependent from external Ca^{2+}, the effect of the antagonist could be clearly seen also in this condition. Besides that, it was still possible to detect the dose-response relationship of ethanol addition (interpretation based only on the graphical representation), which showed that yeasts could respond to ethanol stress with an increase of cytosolic Ca^{2+} concentration, even in the absence of external Ca^{2+}. But the fact that the antagonist effect was not visible, suggests that ethanol could promote a Ca^{2+} response for more than one way. In the condition with 10 mM Ca^{2+} it appeared that the glucose-sensing GPCR was involved in the response to the ethanol shock, with Ca^{2+} coming from an extracellular origin. But, when extracellular Ca^{2+} wasn't present, an alternative pathway through which ethanol could increase cytosolic Ca^{2+} concentration (released from the vacuole) should exist. Summarizing, we can hypothesize that ethanol, in *S. bayanus*, could act by two different pathways: one is the glucose-sensing GPCR pathway (needs extracellular Ca^{2+}), and the other is an alternative pathway (only depends on the Ca^{2+} release from the vacuole).

Since most regressions for *S. cerevisiae* neotype strain (PYCC 4455) weren't significantly linear, the results will be discussed based only on the graphical representation. The results for this species (Fig. 24 B) presented a similar pattern to *S. bayanus*, except in the 0 mM Ca^{2+} condition, where fluorescence variation for 0 mM mannose condition was higher than 200 and 400 mM mannose conditions, when 16% ethanol were added. This shows that, in this species, the antagonist effect was visible even in the absence of external Ca^{2+}, when high ethanol concentrations were added to the cells, meaning that this GPCR could also be activated in situations without external Ca^{2+}.

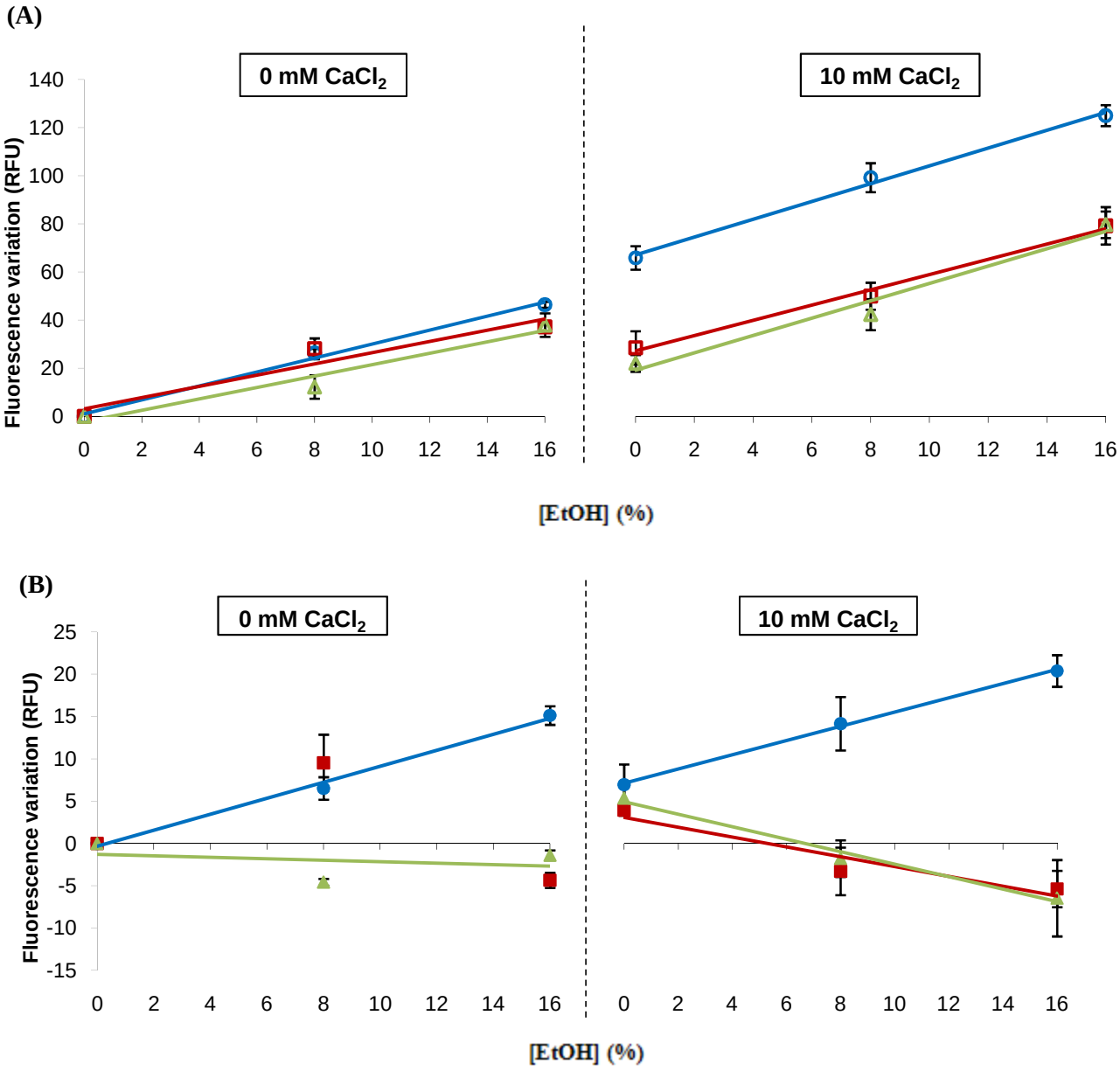


Fig. 24. Influence of ethanol and mannose addition in *S. bayanus* and *S. cerevisiae* neotype strain Ca^{2+} response, in order to test if ethanol is an agonist of the glucose-sensing GPCR.
(A) *S. bayanus* and (B) *S. cerevisiae* neotype strain (PYCC 4455) values of fluorescence variation, after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae)* of electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v), and after addition of 0 (○,●), 200 (□, ■), or 400 mM mannose (△, ▲). The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations, with or without addition of 10 mM $CaCl_2$. The experiments were performed in triplicate for *S. bayanus* and duplicate for *S. cerevisiae*, and the ±standard errors of the mean (SEM) are shown. The response was significantly linear for all conditions of *S. bayanus* ($p< 0.01$ in linearity test; $p>0.05$ in deviation from linearity test), except with addition of 200 mM mannose and 0 mM $CaCl_2$ ($p<0.05$ in deviation from linearity test). The response wasn't significantly linear for all conditions of *S. cerevisiae* ($p>0.05$ in linearity test), except with addition of 0 mM mannose and both $CaCl_2$ concentrations ($p<0.01$). Only the slopes for *S. bayanus* with addition of 0 mM mannose and 10 mM $CaCl_2$, and *S. cerevisiae* with addition of 0 mM mannose and 10 mM $CaCl_2$ were significantly different from zero ($p<0.05$).
S. bayanus linear regression equations for 0 mM $CaCl_2$ are y = 2.8989x + 1.0734; y = 2.3272x + 3.1684; y = 2.3722x - 2.2459, for 0, 200 and 400 mM mannose, respectively. *S. bayanus* linear regression equations for 10 mM $CaCl_2$ are y = 3.6946x + 67.107; y = 3.1694x + 27.194; y = 3.5989x + 19.215, for 0, 200 and 400 mM mannose, respectively. *S. cerevisiae* linear regression equations for 0 mM $CaCl_2$ are y = 0.9457x - 0.3498; y = -0.2723x + 3.9036; y = -0.0874x - 1.2966, for 0, 200 and 400 mM mannose, respectively. *S. cerevisiae* linear regression equations for 10 mM $CaCl_2$ are y = 0.8402x + 7.1146; y = -0.5819x + 3.074; y = -0.7385x + 4.9392, for 0, 200 and 400 mM mannose, respectively.

Unlike *S. bayanus*, antagonist effect in *S. cerevisiae* was higher (fluorescence variation decreases) when agonist concentration increased, in the condition with 10 mM Ca^{2+}, being a pattern already visible in the glucose experiment. This type of response points again to mannose as being an uncompetitive antagonist in this species.

In *S. bayanus*, as happened in the glucose experiments, fluorescence variation values when mannose was added were higher with increasing agonist concentrations. This was again in agreement with mannose being a competitive antagonist, in *S. bayanus*. As refered previously, this is a point to analyze further in future experiments.

The experiment with BY deletion strains for both GPCRs and Ca^{2+} transporters will also help to elucidate some of these results.

3.9. S. cerevisiae BY wild type and deletion strains experiments

The final set of experiments was performed using *S. cerevisiae* BY wild type and deletion strains, in order to investigate further the origin of the Ca^{2+} flux in the response to an ethanol shock, and to determine if ethanol is acting through activation of one or both GPCR systems.

All *S. cerevisiae* BY wild type and deletion strains had similar growth curves, in the condition without ethanol in the growth medium (Fig. 25). Comparing with the neotype strain, it was visible a pronounced difference in the specific growth rates, with the BY strains having higher specific growth rate values.

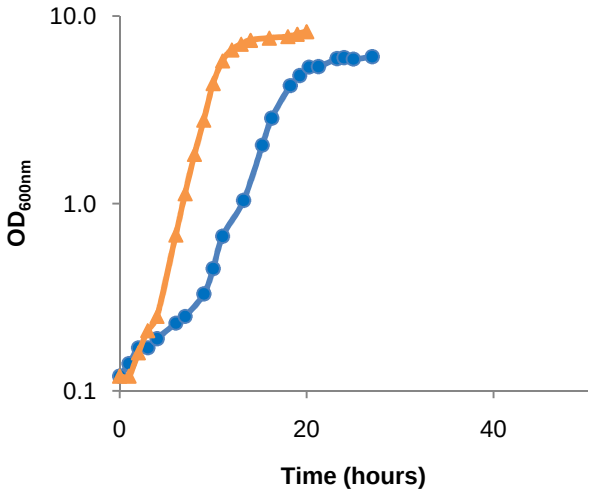


Fig. 25. Growth curves for *S. cerevisiae* BY wild type and deletion strains, and *S. cerevisiae* neotype strain.
Growth curves for *S. cerevisiae* BY wild type and deletion strains (▲) and for *S. cerevisiae* neotype strain (●) growing with 0% ethanol (v/v) in the growth medium. Specific growth rates were 0.48 h^{-1} for all *S. cerevisiae* BY wild type and deletion strains, and 0.29 h^{-1} for *S. cerevisiae* neotype strain. Cells were grown in YEPG liquid medium, pH 5.5, 30ºC, 250 rpm, with an initial OD_{600nm} of 0.1. Logarithmic scale was applied to yy axis.

It was also necessary to optimize the electroporation protocol, in terms of Fluo-4 AM concentration and number of milliseconds used. The optimization was only performed for the wild type BY strain, and the optimized conditions were used for the remaining BY strains in the following experiments. The electroporation conditions that gave the best fluorescence intensity values were 25 ms of electroporation with 2500 V/cm, using Fluo-4 AM with a 1:2 dilution factor.

One of the objectives was to determine if ethanol was acting through activation of one or both GPCR systems. In order to do that, *S. cerevisiae* BY wild type strain, and strains with GPR1 (codes for glucose-sensing GPCR) or STE2 (codes for α-factor pheromone signaling GPCR) genes deleted were compared (Fig. 26; Appendix 9 for fluorescence intensity values). Since most regressions weren't significantly linear ($p>0.05$), the results will be discussed based on the graphical representation. The expected increase in fluorescence variation with increasing ethanol concentrations were only verified in the 10 mM Ca^{2+} condition (slopes significantly different from zero, with $p<0.05$), which seemed to point to a dependence on extracellular Ca^{2+}. If ethanol was acting through any of these GPCRs, it was expected that when the relevant GPCR was deleted, the Ca^{2+} response should be abolished. But, this didn't happened, with intracellular Ca^{2+} concentration increasing in all strains, with increasing ethanol shock concentrations. These results indicated that ethanol doesn't seem to act through glucose-sensing or pheromone signaling GPCR, but through an alternative pathway.

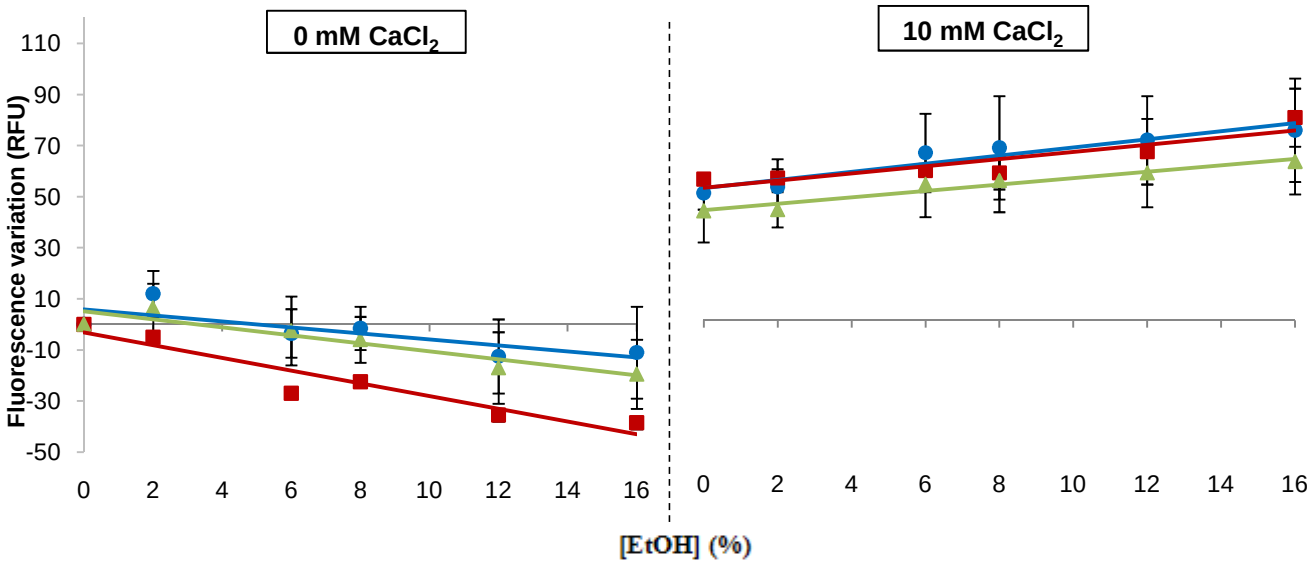


Fig. 26. Ca^{2+} response to ethanol shock of *S. cerevisiae* BY wild type strain and deletion strains (for glucose-sensing and pheromone signaling GPCRs).
S. cerevisiae BY wild type strain (●), BY strain with GPR1 gene deleted (■) and BY strain with STE2 gene deleted (▲) values of fluorescence variation, after 25 ms of electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v). The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations, with or without addition of 10 mM $CaCl_2$. The experiments were performed in duplicate, and the ±standard errors of the mean (SEM) are shown. The response wasn't significantly linear for all conditions ($p>0.05$ in linearity test), except for GPR1 deletion strain with 10 mM $CaCl_2$ ($p<0.05$ in linearity test; $p>0.05$ in deviation from linearity test). All slopes were significantly different from zero ($p<0.05$), except for wild type strain with 0 mM $CaCl_2$ ($p>0.05$).
S. cerevisiae linear regression equations for 0 mM $CaCl_2$ are $y = -3,6143x + 9,9$; $y = -7,9857x + 6,5333$; $y = -4,9x + 10,733$, for BY wild type strain, strain with GPR1 gene deleted and strain with STE2 gene deleted, respectively. *S. cerevisiae* linear regression equations for 10 mM $CaCl_2$ are $y = 5,2143x + 46$; $y = 4,3714x + 47,7$; $y = 4,0714x + 38,833$, for BY wild type strain, strain with GPR1 gene deleted and strain with STE2 gene deleted, respectively.

The other objective was to determine the origin of Ca^{2+} flux in the response to an ethanol shock. In order to do that, *S. cerevisiae* BY wild type strain, and strains with CCH1 (codes for one of the subunits of the plasma membrane Ca^{2+} influx channel), MID1 (codes for the other

subunit of the plasma membrane Ca^{2+} influx channel) or YVC1 (codes for the vacuolar membrane Ca^{2+} channel) genes deleted were compared (Fig. 27; Appendix 9 for fluorescence intensity values). Again, the expected increase in fluorescence variation with increasing ethanol concentrations were only verified in the 10 mM Ca^{2+} condition (slopes for wild type and MID1 deletion strain were significantly different from zero, $p<0.01$), pointing to a dependence on extracellular Ca^{2+}. If the response to an ethanol shock was dependent from Ca^{2+} coming from extracellular or intracellular sources, it was expected that when the relevant Ca^{2+} channel was deleted, the Ca^{2+} response should be abolished.

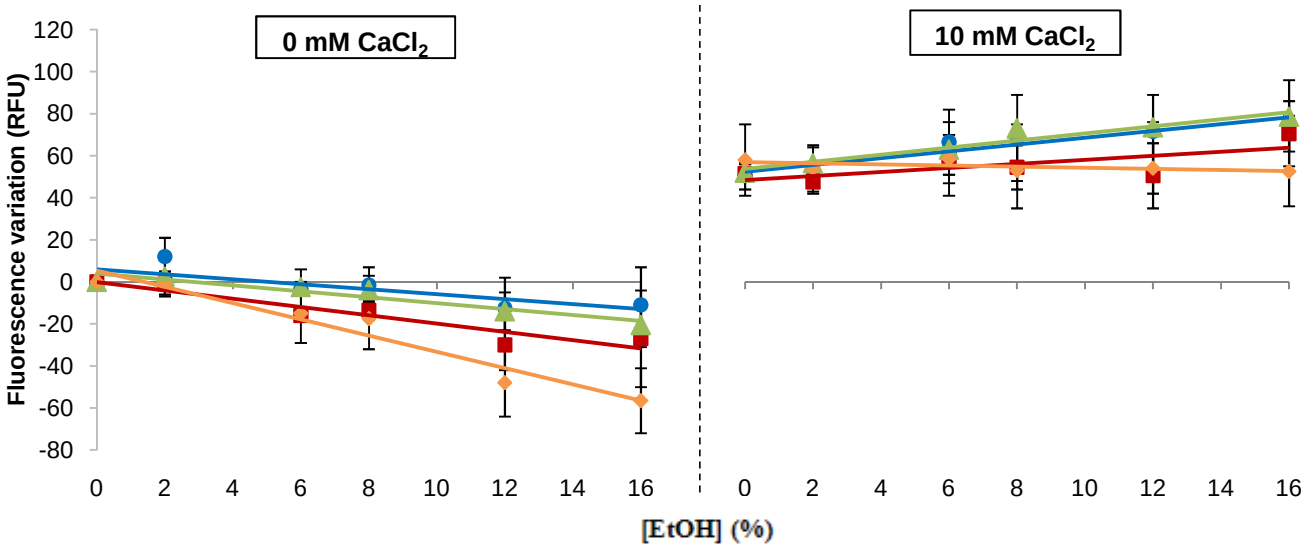


Fig. 27. Ca^{2+} response to ethanol shock of *S. cerevisiae* BY wild type strain and deletion strains (for Ca^{2+} transporters in plasma and vacuolar membranes).
S. cerevisiae BY wild type strain (●), BY strain with CCH1 gene deleted (■), BY strain with MID1 gene deleted (▲) and BY strain with YVC1 gene deleted (♦) values of fluorescence variation, after 25 ms of electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v). The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations, with or without addition of 10 mM $CaCl_2$. The experiments were performed in duplicate, and the ±standard errors of the mean (SEM) are shown. The response wasn't significantly linear ($p>0.05$ in linearity test), except for CCH1 deletion strain with both $CaCl_2$ concentrations, and YVC1 deletion strain with 0 mM $CaCl_2$ ($p<0.05$ in linearity test and $p>0.05$ in deviation from linearity test).The slopes for wild type strain with 0 mM $CaCl_2$, and CCH1 and YVC1 deletion strains with 10 mM $CaCl_2$ weren't significantly different from zero ($p>0.05$).
S. cerevisiae linear regression equations for 0 mM $CaCl_2$ are y = -3,6143x + 9,9; y = -6,2714x + 7,3667; y = -4,3857x + 8,9333; y = -12,171x + 19,6, for BY wild type strain, strain with CCH1 gene deleted, strain with MID1 gene deleted and strain with YVC1 gene deleted, respectively. *S. cerevisiae* linear regression equations for 10 mM $CaCl_2$ are y = 5,2143x + 46; y = 2,8571x + 45,5; y = 5,5286x + 46,733; y = -0,9429x + 58,3, for BY wild type strain, strain with CCH1 gene deleted, strain with MID1 gene deleted and strain with YVC1 gene deleted, respectively.

Comparing with the wild type, in the strain with the YVC1 gene deleted (Fig. 27), the Ca^{2+} response to the increase in ethanol shock concentration was abolished (slope wasn't significantly different from zero, with $p>0.05$), which indicated that Ca^{2+} released from the vacuole, by the Yvc1p channel in the vacuolar membrane, was essential to the yeast's response to an ethanol shock. But, the same has happened in the strain with the CCH1 gene deleted (slope wasn't significantly different from zero, with $p>0.05$), that codes for the putative pore-forming subunit of the plasma membrane Ca^{2+} channel. The other subunit of the same channel (Mid1p) codes for a plasma membrane protein involved in pheromone-

stimulated Ca^{2+} uptake[67]. Based on this information, and in the fact that the deletion of the CCH1 gene caused the abolishment of the Ca^{2+} response, but not when MID1 gene was deleted, we can hypothesize that Cch1p was the main subunit involved in the Ca^{2+} response to an ethanol shock.

These results show that *S. cerevisiae* respond to an ethanol shock with an increase of cytosolic Ca^{2+} concentration, and Ca^{2+} seems to come both from extracellular media and the intracellular store (vacuole). A similar response to an external stress has already been reported for yeasts responding to hypotonic shock. This stress generated a Ca^{2+} pulse, that was primarily generated from intracellular stores. Only after this first response, was that the sustained increase in cytosolic Ca^{2+} concentration depended upon Ca^{2+} entering from the extracellular medium[5]. In the present experiment, it wasn't determined which source of Ca^{2+} is first activated, but we can hypothesize that a similar response is happening with ethanol shock, with Ca^{2+} coming from different sources, in different stages of the response.

In a future work, more replicates and different experimental conditions are needed in order to try to explain the decreasing of fluorescence variation values in the 0 mM Ca^{2+} condition.

4. Conclusions

This work had several main objectives. One was to investigate if *S. bayanus* and *S. cerevisiae* neotype strain (PYCC 4455) respond to ethanol shock with an increase of cytosolic Ca^{2+} concentration. This was verified for both species, but *S. cerevisiae* had the highest increase in fluorescence variation per cell with Fluo-4 AM, probably because of their lower natural resistance to ethanol. The rise of cytosolic Ca^{2+} levels will result in the formation of Ca^{2+}/calmodulin complexes that will activate calcineurin. When activated, calcineurin dephosphorylates the transcription factor Crz1p, causing its rapid translocation to the nucleus, where it's responsible for the expression of genes that induce adaptive tolerance to ethanol[2]. Since *S. cerevisiae* is less naturally resistant to ethanol, it needs to have a stronger response to ethanol shock, in terms of Ca^{2+} concentration increase.

Other goal was to detect the origin of the Ca^{2+} involved in this response. The results for both *S. bayanus* and *S. cerevisiae* neotype strain (PYCC 4455) seems to indicate that the main source of Ca^{2+} for this type of response is the intracellular stores, that in the case of *S. cerevisiae* is considered the vacuole[31]. Unlike the response to glucose[43,56], the external Ca^{2+} didn't seem to have an important contribution for the ethanol shock response. But, with the *S. cerevisiae* BY wild type and deletion strains experiments, it seems that these species respond to an ethanol shock with an increase of cytosolic Ca^{2+} concentration, and Ca^{2+} seems to come both from extracellular media and the intracellular store (vacuole). We can hypothesize that Ca^{2+} is coming from different sources, in different stages of the response, as happen in the yeast's response to a hypotonic shock[5].

Other main objective was to investigate if growing both species in the presence of ethanol had some influence in the following increase of cytosolic Ca^{2+} concentration in response to an ethanol shock. Since pre-exposure to ethanol can activate stress response mechanisms that results in a transient resistance to higher levels of ethanol[1], it's hypothesized that this could lead to a different pattern of Ca^{2+} response. For *S. bayanus*, growth with 0, 3 or 9% ethanol leaded to similar patterns. The results for *S. cerevisiae* neotype strain show that cells grown with 3% ethanol seemed to respond in a more intense way to the ethanol shock, than cells grown without ethanol in the growth medium.

The last main objective was to investigate if the increase in cytosolic Ca^{2+} concentration is due to the activation of the glucose-sensing GPCR by ethanol, that in this case will be acting as an agonist. In *S. bayanus* and *S. cerevisiae* neotype strain, ethanol seemed to act as an agonist of this GPCR, because it was visible a dose-response effect and also because mannose seemed to act as antagonist. But, the results from *S. cerevisiae* BY wild type and deletion strains didn't support these claims, because the deletion of the gene coding for the glucose-sensing GPCR (and also of the gene coding for the pheromone signaling GPCR) didn't abolished the Ca^{2+} signalling in response to an ethanol shock. These results suggest that ethanol wasn't acting through glucose-sensing or pheromone signaling GPCR, but through an alternative pathway, to promote an increase of cytosolic Ca^{2+} concentration in yeasts.

5. References

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6. Appendix

Appendix 1. *S. bayanus* values of fluorescence intensity (exc=494 nm; em=516 nm), after 10, 20, 25 or 30 ms of electroporation with 2500 V/cm, or 12 ms with 5000 V/cm. The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations (v/v), with or without addition of 10 mM $CaCl_2$. The average and ±standard errors of the mean (SEM) values from three replicates are indicated.

[$CaCl_2$]	[EtOH] shock	10 ms		20 ms		25 ms		30 ms		5000 V/cm	
		Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM
0 mM	0%	26.0	1.9	43.2	3.6	55.0	2.8	63.6	4.6	82.4	2.6
	2%	33.4	2.0	53.4	2.7	66.7	5.1	70.2	12.1	106.8	1.3
	6%	39.2	3.4	64.1	4.2	79.6	3.7	90.1	2.9	119.4	3.4
	8%	46.3	1.2	60.7	2.7	80.6	4.5	88.8	10.2	122.7	1.0
	12%	51.9	3.6	73.3	4.3	91.2	4.1	100.8	2.5	144.7	10.3
	16%	56.0	1.0	73.4	5.1	94.9	8.5	108.6	1.8	155.2	4.8
10 mM	0%	56.7	0.9	114.4	2.8	150.2	6.6	171.8	11.0	222.0	8.0
	2%	66.4	3.3	122.2	4.5	161.7	0.9	183.8	10.8	245.5	6.0
	6%	70.4	0.7	134.3	6.5	173.7	1.8	205.2	11.5	266.7	10.2
	8%	72.4	0.0	136.7	4.0	182.8	8.7	205.0	11.1	278.4	11.1
	12%	74.8	4.3	139.0	4.9	189.6	7.6	213.3	10.6	280.9	11.9
	16%	86.2	2.7	146.5	7.5	198.2	9.2	221.6	8.9	301.6	8.6

Appendix 2. *S. bayanus* values of fluorescence intensity per cell with Fluo-4 AM, after 20, 25 or 30 ms of electroporation with 2500 V/cm. The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations (v/v), without addition of $CaCl_2$. The average and ±standard errors of the mean (SEM) values from three replicates are indicated.

[$CaCl_2$]	[EtOH] shock	20 ms		25 ms		30 ms	
		Average	SEM	Average	SEM	Average	SEM
0 mM	0%	0.46	0.06	0.58	0.06	0.49	0.00
	2%	0.53	0.08	0.68	0.08	0.52	0.08
	6%	0.65	0.07	0.90	0.10	0.72	0.02
	8%	0.62	0.06	0.88	0.09	0.76	0.07
	12%	0.73	0.08	1.09	0.11	0.80	0.02
	16%	0.77	0.17	1.12	0.18	0.90	0.05

Appendix 3. *S. cerevisiae* values of fluorescence intensity (exc=494 nm; em=516 nm), after 5, 10 ms or 15 ms of electroporation with 2500 V/cm. The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations (v/v), with or without addition of 10 mM $CaCl_2$. The experiments were performed once.

[$CaCl_2$]	[EtOH] shock	5 ms	10 ms	15 ms
0 mM	0%	19.8	9.1	-7.8
	2%	17.1	11.8	-7.2
	6%	17.2	15.2	1.3
	8%	15.4	17.0	3.3
	12%	19.4	21.9	6.4
	16%	23.8	20.1	10.6
10 mM	0%	7.7	14.3	-1.4
	2%	4.9	19.2	-3.4
	6%	15.0	20.1	9.5
	8%	17.6	20.5	9.2
	12%	22.3	26.6	13.6
	16%	26.2	29.1	15.6

Appendix 4. *S. cerevisiae* values of fluorescence intensity per cell with Fluo-4 AM, after 5, 10 or 15 ms of electroporation with 2500 V/cm. The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations (v/v), without addition of $CaCl_2$. The experiments were performed once.

[$CaCl_2$]	[EtOH] shock	5 ms	10 ms	15 ms
0 mM	0%	0.79	0.26	-0.38
	2%	1.42	0.58	-0.57
	6%	1.52	0.86	0.11
	8%	1.39	0.94	0.28
	12%	2.07	1.47	0.57
	16%	1.48	1.28	0.77

Appendix 5. *S. bayanus* and *S. cerevisiae* values of fluorescence intensity (exc=494 nm; em=516 nm), after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae*) electroporation with 2500 V/cm, for cells grown in the presence of 0, 3 or 9% ethanol (v/v). The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations (v/v), with or without addition of 10 mM $CaCl_2$. The average and ±standard errors of the mean (SEM) values from six replicates for *S. bayanus* grown with 0% ethanol and three replicates for the remaining conditions are indicated.

		S. bayanus						*S. cerevisiae*			
[$CaCl_2$]	[EtOH] shock	0% ethanol in growth medium		3% ethanol in growth medium		9% ethanol in growth medium		0% ethanol in growth medium		3% ethanol in growth medium	
		Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM
0 mM	0%	61.7	5.0	48.8	8.5	84.5	0.8	8.5	2.1	-12.5	4.8
	2%	75.1	5.4	56.5	9.8	102.8	1.8	14.8	1.6	-3.7	4.3
	6%	82.5	4.0	65.2	12.7	114.4	5.7	12.9	3.5	-1.0	4.5
	8%	87.4	5.2	81.2	12.1	128.1	9.2	15.0	3.3	-1.1	3.0
	12%	93.7	4.4	79.4	11.4	140.8	6.1	20.5	2.6	5.6	4.6
	16%	102.3	8.2	87.1	15.7	154.0	6.7	23.7	2.0	8.0	3.0
10 mM	0%	150.6	10.2	119.2	11.3	176.5	8.7	15.5	3.7	-6.1	1.9
	2%	161.1	10.0	131.8	12.2	196.8	3.0	18.1	5.3	-2.8	2.7
	6%	173.8	9.7	145.9	11.4	210.0	6.2	21.8	5.2	-1.7	2.2
	8%	182.0	11.3	147.1	10.2	215.5	4.3	22.7	5.3	0.0	2.8
	12%	187.8	12.5	148.1	13.1	226.8	1.3	22.3	5.3	3.4	4.3
	16%	194.9	12.9	164.6	7.7	237.8	4.4	28.9	3.3	8.1	3.3

Appendix 6. *S. bayanus* and *S. cerevisiae* values of fluorescence intensity per cell with Fluo-4 AM (exc=494 nm; em=516 nm), after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae*) electroporation with 2500 V/cm, for cells grown in the presence of 0, 3 or 9% ethanol (v/v). The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations (v/v), with or without addition of 10 mM $CaCl_2$. The average and ±standard errors of the mean (SEM) values from three replicates are indicated.

		S. bayanus						*S. cerevisiae*			
[$CaCl_2$]	[EtOH] shock	0% ethanol in growth medium		3% ethanol in growth medium		9% ethanol in growth medium		0% ethanol in growth medium		3% ethanol in growth medium	
		Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM
0 mM	0%	0.39	0.06	0.35	0.07	0.37	0.04	0.20	0.02	-1.52	0.68
	2%	0.50	0.07	0.44	0.12	0.42	0.05	0.68	0.19	-0.25	1.21
	6%	0.54	0.09	0.50	0.15	0.49	0.02	0.60	0.16	-0.14	1.23
	8%	0.57	0.07	0.65	0.14	0.57	0.07	0.86	0.21	0.48	1.15
	12%	0.60	0.06	0.60	0.15	0.62	0.06	1.08	0.29	1.24	1.05
	16%	0.72	0.11	0.61	0.14	0.77	0.07	1.88	1.06	2.87	1.36
10 mM	0%							0.27	0.01	-0.97	0.26
	2%							0.59	0.07	-0.38	0.60
	6%							0.88	0.06	0.12	0.86
	8%							0.95	0.12	0.30	0.89
	12%							1.14	0.36	0.97	1.36
	16%							1.76	0.63	3.16	1.65

Appendix 7. *S. bayanus* and *S. cerevisiae* values of fluorescence intensity (exc=494 nm; em=516 nm), after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae*) electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v), and after addition of 0, 200, or 400 mM mannose. The autofluorescence values were subtracted to all results, and then presented as a function of increasing glucose concentrations, with or without addition of 10 mM $CaCl_2$. The average and ±standard errors of the mean (SEM) values from three replicates for *S. bayanus* and two replicates for *S. cerevisiae* are indicated.

		S. bayanus						***S. cerevisiae***					
[$CaCl_2$]	**[glucose]**	**0 mM mannose**		**200 mM mannose**		**400 mM mannose**		**0 mM mannose**		**200 mM mannose**		**400 mM mannose**	
		Average	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**
0 mM	**0 mM**	72.72	8.98	103.73	6.09	99.37	6.39	0.94	2.96	-0.76	0.78	-3.81	1.79
	50 mM	83.98	5.29	118.97	5.65	110.68	3.98	0.85	9.86	-6.52	0.60	-1.55	2.12
	100 mM	82.73	7.66	117.11	3.77	116.88	2.73	7.75	1.11	-4.29	0.73	-11.95	4.73
	300 mM	85.54	6.21	119.24	3.38	118.67	4.49	11.86	1.27	-3.34	1.23	-3.13	1.26
10 mM	**0 mM**	138.56	9.26	132.21	12.98	121.43	9.47	16.44	0.80	3.17	1.41	1.54	0.12
	50 mM	147.14	10.17	149.59	3.52	136.75	3.93	16.78	5.97	4.83	0.99	-6.95	1.10
	100 mM	142.24	12.72	148.49	7.31	134.88	3.54	11.83	0.01	-2.92	1.04	-11.46	2.96
	300 mM	134.78	13.09	145.32	4.83	138.42	4.92	11.75	0.32	-11.21	3.43	-9.36	1.75

Appendix 8. *S. bayanus* and *S. cerevisiae* values of fluorescence intensity (exc=494 nm; em=516 nm), after 25 (for *S. bayanus*) or 10 ms (for *S. cerevisiae*) of electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v), and after addition of 0, 200, or 400 mM mannose. The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations, with or without addition of 10 mM $CaCl_2$. The average and ±standard errors of the mean (SEM) values from three replicates for *S. bayanus* and two replicates for *S. cerevisiae* are indicated.

		S. bayanus						***S. cerevisiae***					
[$CaCl_2$]	**[EtOH] shock**	**0 mM mannose**		**200 mM mannose**		**400 mM mannose**		**0 mM mannose**		**200 mM mannose**		**400 mM mannose**	
		Average	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**
0 mM	**0%**	72.72	8.98	103.73	6.09	99.37	6.39	0.00	0.00	0.00	0.00	0.00	0.00
	8%	99.13	7.47	131.86	5.15	111.61	1.87	6.52	1.33	9.53	3.34	-4.59	0.40
	16%	119.10	10.23	140.97	6.97	137.33	3.55	15.13	1.10	-4.36	0.88	-1.40	0.58
10 mM	**0%**	138.56	9.26	132.21	12.98	121.43	9.47	6.95	2.40	3.93	0.63	5.34	1.67
	8%	171.91	9.20	153.71	4.05	141.69	6.14	14.16	3.16	-3.30	2.81	-1.78	2.16
	16%	197.67	7.03	182.92	13.85	179.01	11.81	20.39	1.87	-5.38	2.16	-6.47	4.52

Appendix 9. *S. cerevisiae* BY wild type and deletion strains values of fluorescence intensity (exc=494 nm; em=516 nm), after 25 ms of electroporation with 2500 V/cm, for cells grown in the presence of 0% ethanol (v/v). The autofluorescence values were subtracted to all results, and then presented as a function of increasing ethanol shock concentrations, with or without addition of 10 mM $CaCl_2$. The average and ±standard errors of the mean (SEM) values from two replicates are indicated.

				Gene deleted									
[$CaCl_2$]	**[EtOH] shock**	**Wild type**		**GPR1**		**STE2**		**CCH1**		**MID1**		**YVC1**	
		Average	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**
0 mM	**0%**	97,5	30,5	106,5	23,5	135,5	27,5	85,0	60,0	161,0	33,0	86,5	38,5
	2%	109,5	39,5	101,5	24,5	142,0	37,0	84,0	54,0	163,5	24,5	85,5	43,5
	6%	94,0	40,0	79,5	21,5	133,0	41,0	69,0	59,0	158,5	30,5	71,5	52,5
	8%	96,0	39,0	84,0	25,0	129,5	36,5	71,5	64,5	157,0	40,0	69,0	53,0
	12%	85,0	45,0	71,0	26,0	118,5	41,5	55,0	72,0	147,0	42,0	38,5	54,5
	16%	86,5	48,5	68,0	26,0	116,0	41,0	58,0	83,0	140,5	43,5	30,0	54,0
10 mM	**0%**	148,0	37,0	162,5	25,5	179,0	40,0	136,5	58,5	213,0	29,0	144,5	55,5
	2%	150,5	41,5	163,0	27,0	179,5	34,5	132,5	62,5	217,5	33,5	140,5	49,5
	6%	164,0	46,0	166,0	25,0	189,5	40,5	143,5	71,5	224,0	33,0	145,0	56,0
	8%	166,0	51,0	165,0	30,0	191,0	40,0	139,5	70,5	234,0	31,0	139,5	56,5
	12%	169,0	48,0	173,5	36,5	194,0	41,0	135,5	75,5	234,5	35,5	140,5	50,5
	16%	173,0	51,0	187,0	35,0	198,5	40,5	155,5	68,5	239,5	40,5	139,0	55,0